REGULATION OF HAEMATOPOIETIC STEM CYCLE (CFU-S) PROLIFERATION IN IRRADIATED MICE

Abdul Rabbi Manaf Ali

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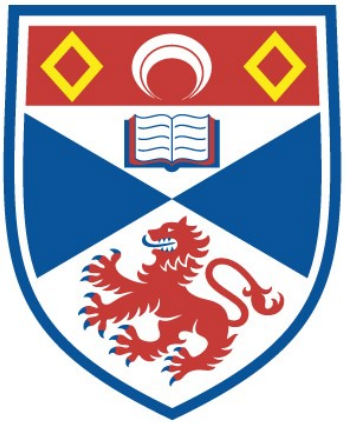
University of St Andrews



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A Thesis

Submitted to the University of St. Andrews for the degree of Doctor of Philosophy

by

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ABSTRACT

It has been suggested that the proliferation of haematopoietic stem cells (CFU-S) in mice is controlled by the balance of inhibitory and stimulatory factors. In normal mice about 10 percent of the CFU-S population are in DNA synthesis. It has been suggested that a high concentration of inhibitor blocks CFU-S from entering into DNA synthesis. Following damage by cytotoxic agents such as drugs or irradiation about 30 - 50 percent of CFU-S were in DNA synthesis and also stimulator was shown to be present. In this study the entry of CFU-S into DNA synthesis following low and sub-lethal doses of whole body X-irradiation has been studied. Furthermore the stimulator producing cells were also characterized.

The number of CFU-S in bone marrow was not affected following exposure to a dose of 0.5 Gy. However the number of committed progenitors for the granulocyte/macrophage lineage was significantly reduced. The percentage of CFU-S in DNA synthesis was found to increase to 37.0 ± 7.0 percent at 30 minutes and 43.9±11.2 percent at 2 hours from that observed in unirradiated mice. However at 6 hours the percentage was 14.8 8.1 percent. At a sub-lethal dose of 4.5 Gy, the

percentage of CFU-S in DNA synthesis increased to 34.0±14.0 percent at 6 hours after exposure, however before this time the percentage remained at a similar level to unirradiated control mice. When plugs of bone marrow were irradiated in-vitro at 0.5 Gy and 4.5 Gy doses, the time of CFU-S entering into DNA synthesis was the same as following in-vivo irradiation.

The dose response curve of CFU-S entering into DNA synthesis when measured at 2 hours after exposure showed that the percentage was increased as the dose was increased and reached 30-50 percent at a dose of 0.5 Gy. Above this dose the CFU-S population was not stimulated at this time. When the percentage of CFU-S in DNA synthesis was measured at 6 hours after exposure, the values were the same as control for doses less than 0.5 Gy and above this dose the values were 30-50 percent. The presence of stimulator in bone marrow after irradiation was found to parallel the proliferative activity of CFU-S. The CFU-S population obtained 1 hour after 1.5 Gy was shown not to respond to stimulator as CFU-S from normal bone marrow did.

The conditioned media prepared from bone marrow of mice irradiated at 9.0 Gy (1 to 5 days post irradiation) increased the proportion of CFU-S from normal bone marrow in DNA synthesis to 30-50 percent. The depletion of $Thy1.2^+$ cells from regenerating bone marrow did not affect the ability to produce stimulator. However when Fc^+ and $Ia\text{-}2k^+$ cells were removed the stimulator production was affected. This suggests that the stimulator producing cells were radioresistant, $Thy1.2^-$, Fc^+ and $Ia\text{-}2k^+$.

DECLARATION

I hereby declare that the research reported in this thesis is in fulfilment of the requirements for the degree of Doctor Philosophy. The results of work were done by me during my period of matriculation for the above degree. No part of this work has been submitted previously for a higher degree.

The research was conducted in the Department of Anatomy and Experimental Pathology, United College of St. Salvator and St. Leonard, University of St. Andrews, under supervision of Dr. A.C. Riches and Dr. E. G. Wright.

CERTIFICATE

We hereby certify that Abd. Rabbi Manaf Ali has spent nine terms engaged in research work under our direction, and that he has fulfilled the conditions of General Ordinance No. 12 (Resolution of the University Court No. 1, 1967), and that he is qualified to submit the accompaning thesis for the Degree of Doctor of Philosophy.

ACADEMIC RECORD

I graduated from the National University of Malaysia in July 1979 with degree of Bachelor of Science (Biochemistry). I was awarded a Master of Science in Radiation Biophysics from University of Dundee in December 1981.

I matriculated as a postgraduate research student in the Department of Anatomy and Experimental Pathology, Unversity of St. Andrews in Jannuary 1981.

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ABBREVIATION AND ALTERNATIVE NAMES

ARA-C:	cytosine arabinoside
B-CFC:	B-lymphocyte colony forming cells
BFU-E:	Burst colony forming units- erythroid
BPA:	Burst promoting activity
BSA:	Bovine serum albumin
CBME:	Crude bone marrow extract
CFU-C:	Colony forming units- culture
CFU-E:	Colony forming units- erythroid
CFU-S:	Colony forming units-spleen
CM:	Conditioned media
CML:	Chronic myeloid leukemia
CSA:	Colony stimulating activity
D_{37}/ Do:	Dose which reduces survival to 37 percent when survival is exponential with dose
FCFC:	Fibroblastic colony forming cells
Fc^{+}-CM:	Conditioned media of rosettes fraction
Fc^{-}-CM:	Conditioned media of non-rosettes fraction
GM-CFC:	Granulocyte/macrophage colony forming cells
G-6-PD:	Glucose-6-phosphate dehydrogenase
^{3}HTDR:	Tritiated thymidine

CBA/H-CM: Conditioned media prepared after cytolysis of Ia-$2k^+$ cells.

IMS: Isopropyl methane sulphonate

LPS: Lipopolysaccharide

NBME: Normal bone marrow extract

PEC-CM: Peritoneal exudate cells - conditioned media

PHA: Phytohaemagglutinin

RBME: Regenerating bone marrow extract

SRBC: Sheep red blood cells

Thy1.2^--CM: Conditioned media prepared after the removal of Thy1.2^+ cells.

TCGF: T - cell growth factors

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CHAPTER 1

INTRODUCTION

The mature blood cells are usually divided into three broad classes: red cells, white cells and platelets. They are the end cells of the haematopoietic system. Most of these cells have a limited life span, are highly differentiated and incapable of proliferation (with the exception of the lymphocytes). The continuous production of blood cells therefore requires a hierachy of cells which can generate the mature blood cells in each class of blood cells. The existence of stem cells which can differentiate along the main cell lines and be capable of maintaining their own number is vital in supporting a haematopoietic system (Lajtha, 1979).

1.1 Haematopoietic stem cells

From the study of the clonal expansion of marked cells by using chromosome and genetic markers, most of the mature elements of lymphoid and myeloid systems were shown to be derived from a common pool of stem cells, both in the mouse (Micklem and Loutit, 1966) and in man (Fialkow et al. 1978; Prachal et al. 1978).

In the mouse it was known that following the damage induced in the myeloid and lymphoid tissues by radiation recovery would occur when injected with suspensions of bone marrow cells (Lorenz and Congdon, 1954). Micklem and Loutit (1966) used the T6 chromosome marker system to study the repopulation of donor cells in the lymphoid and myeloid elements. The T6 chromosome is one of the products of unequal translocation of two medium size autosomes in the irradiated male mouse. It can be distinguished from the other chromosomes because of its small size. Micklem and Loutit (1966) found that both lymphoid and myeloid tissues were repopulated by the bone marrow cells with this discrete marker.

1.1.1 Pluripotent and restricted stem cells

Evidence for a pluripotent stem cell giving rise to lymphoid and myeloid cells as well as restricted stem cells giving rise to lymphoid and myeloid cells was demonstrated in a series of experiments using radiation induced chromosome abnormalities as markers (Abramson et al. 1977; Phillip et al. 1978). Chromsome abnormalities in individual cells were induced by exposing the mice to whole body irradiation at a high dose which destroyed most of the haematopoietic cells but among the survival cells in bone marrow a high proportion had abnormal chromosomes. These cells were injected in limiting dilution into sub-lethally irradiated W/W^{v} mice and allowed to grow and differentiate for several months.

In these experiments three functional assays were used to determine the distribution of chromosomally marked cells in the lymphoid and myeloid systems. The spleen colony assay was used for the myeloid cell system and the culture of splenic lymphocytes either with lipopolysaccharide (LPS) or phytohaemagglutinin (PHA) to stimulate the proliferative responses of B and T lymphocytes. The self renwal capacity

of marked stem cells was tested by transplanting bone marrow from reconstituted W/W^V recipients and two months later spleen colonies, PHA blasts and LPS were examined for the presence of chromosome markers.

Three distinct patterns of clones were observed, one included the observation of a unique chromosome marker in CFU-S, PHA and LPS blasts, suggesting a pluripotent stem cell for the myeloid and lymphocyte series. Another included the observation of a unique chromosome marker in CFU-S only and PHA lymphocytes only which suggested they were derived from restricted stem cells for myeloid and for T lymphocytes. However stem cells restricted to the B lymphocyte series were not detected. The presence of stem cells restricted to B and T lymphocytes was suggested by Phillip et al. (1978). In mice repopulated with cells from long term bone marrow cultures maintained for five weeks (Dexter et al. 1978), it was shown that lymphocyte cultures stimulated with concanacalin A or LPS have the same chromsome markers (Phillip et al. 1978)

1.1.2 Colony forming units-spleen (CFU-S)

An in-vivo assay for the detection of a class of haematopoietic stem cells was first developed by Till and McCulloch (1961). Bone marrow cells were injected into lethally irradiated mice and discrete macroscopically visible colonies were observed after 7 to 14 days. Histological examination of these colonies demonstrated that they contained erythroid, granulocytic and megakaryocytic elements. None of the lymphocyte series was observed. Erythroid colonies are detectable histologically by 4 to 5 days within the splenic red pulp and appear as surface colonies as early as day 6 (Moore and Metcalf, 1970). Granulocytic colonies are found along trabeculae of the spleen or in the sub-capsular regions and less frequently within the atrophic lymphoid follicles. The vast majority of granulocytic colonies are neutrophilic which can be detected at 4 days and macroscopically by 7 to 8 days (Moore and Metcalf, 1970). Megakaryocytic colonies grow under the splenic capsule and generally appear as clusters of mature cells (Curry and Trentin, 1967). Mixed colonies are the largest groups observed when mice were assayed for the longer period of time (Siminovitch et al. 1963; Magli et

al. 1982) and most frequently contained erythroid with other types (Moore and Metcalf, 1970). Magli et al. (1982) however have shown that not all the colonies visible at day 7 or 8 will be visible at days 10 or 11 later.

Each of these colonies was shown to be derived from single cells in the inoculum. Chen and Schooley (1968) used a mixture of bone marrow cells with two T6 and one T6 chromosome markers as the donor cells and analysed the karyotype of spleen colonies 10 days after transplantation. The analysis showed that 41 of the colonies were composed wholly of one karyotype either with two T6 or one T6 chromosome and 7 of the colonies showed 92 to 98 percent of one karyotype. In mixed colonies, the erythroblasts and granulocytes were also shown to be derived from a single cell (Wu et al. 1976). Bone marrow cells bearing a unique radiation induced chromosome marker were injected into unirradiated W/W^v anaemic mice as recipients and karyotypic studies of cells from 14 day colonies were made in conjunction with ^{55}Fe autoradiography and peroxidase staining. In one colony, 91 to 100 percent of the mitoses were found to be of a unique type and 10 out of 12 colonies showed the same unique marker in both ^{55}Fe labelled erythroid and peroxidase positive granulocytic metaphases.

The spleen colonies also contain cells which can produce further spleen colonies following transplantation of a suspension of cells derived from one colony into lethally irradiated mice (Siminovitch et al. 1963).

1.1.3 Seeding efficiency of colony forming cells-spleen

The number of colony forming units-spleen (CFU-S) indicates the number of haematopoietic stem cells in an inoculum which settle in the spleen and form surface colonies. Haematopoietic stem cells would also have settled in other haematopoietic tissues such as the bone marrow and some might be lost due to seeding in an unsuitable environment or due to terminal differentiation. Therefore the total number of stem cells in the inoculum can be related to the number of spleen colony forming units by taking into account this fractional seeding using the seeding efficiency or f factor.

The seeding efficiency can be determined by injecting a known number of CFU-S into a primary lethally irradiated recipient. After a few hours, the fraction of CFU-S seeding the spleen can be assayed by a secondary transfer (Siminovitch et al. 1963). The value of the f factor was dependent on the time the CFU-S are assayed in the secondary transfer. After two hours Siminovitch et al. (1963) reported the f factor was 0.17 and after 24 hours the value was 0.08 (Playfair and Cole, 1965). If the primary recipient was irradiated three days previously the value of the f factor would be the same at two and 24 hours (Lahiri et al. 1970). The seeding of CFU-S in the spleen was a function of the space available. Lord (1971) showed the changes of CFU-S number in the primary spleen over the period of 24 hours after irradiation paralleled the post irradiation changes in the size of the spleen.

The f factor also changes when the donor mice are previously treated with various cytotoxic agents. Fred and Smith (1968) reported treatment with endotoxin, vinblastine or sub-lethal irradiation (2 to 3 Gy) 1 to 2 days prior to transfer of the marrow cells reduced the 3 hour f values to 0.12 - 0.08. In a comparative study of the seeding

efficiency of the colony forming cells from normal, regenerating and velocity sedimented cycling and non-cycling marrow, Monette and De Mello (1979) showed that colony forming cells in cycle were found to have a lower seeding efficiency compared to normal bone marrow or sedimented non-cycling cells.

1.1.4 Relationship between CFU-S and the lymphoid system

A close relationship between CFU-S and the lymphoid system has been demonstrated in many studies. Wu et al. (1968) used radiation induced chromosome markers to study this relationship. The unique chromosome markers were shown to be present both in the thymus, bone marrow and spleen colonies of W/W^V unirradiated mice which received bone marrow of irradiated donors (Wu et al. 1968). The same unique chromosome markers were found both in the thymus and spleen colonies. More over it was noted that the cells derived from blood lymphocyte mixed cultures have the same chromosome markers as observed in the spleen colonies of the bone marrow of the irradiated rats (Nowell et al. 1970). The rosette forming cells from the spleen of W/W^V anaemic

mice which were repopulated with bone marrow from irradiated donors also had the same karyotypic markers as cells in spleen colonies (Edward et al. 1971).

Furthermore in a study reported by Metcalf and Johnson (1976), they showed that a certain population of spleen colonies at day seven or later contain a variable number of B-lymphocyte colony forming cells (B-CFC) which could be detected when grown in agar in the presence of 2-mercapto ethanol and sheep erythrocytes. Furthermore Lala and Johnson (1979) showed that the spleen colonies and the B-lymphocyte colony forming cells were of the same karyotypes.

1.1.5 Spatial organization of CFU-S

The distribution of CFU-S in the femoral marrow of the mouse is not entirely homogenous. Lord and Hendry (1975) using different needle sizes were able to sample marrow from different regions of the medullary cavity. The concentration of CFU-S near to the bone surface was 2-3 times higher than of the longitudinal axis of femur (Lord

and Hendry, 1972; Lord et al. 1975). This concentration gradient appears to obey a square law relationship.

1.1.6 Human haematopoietic stem cells

The evidence for the clonality of human haematopoietic stem cells came from studies of pathological conditions. In these studies the clonality of haematopoietic cells was deduced from the Philadelphia (Ph') chromosome or the enzyme glucose-6-phosphate dehydrogenase (G-6-PD). In the patients with chronic myeloid leukemia (CML), the Ph' chromosome was found in cells of the erythrocytic, megakaryocytic, granulocytic and monocytic line (Whang et al. 1963). The Ph' chromosome is an abnormal chromosome developed in patients with CML due to the translocation of the long arms of chromosome 22 to the short arms of chromosome 9 or 2 and other chromosomes (Rowley, 1973; 1978)

The enzyme G-6-PD occurs in two forms of iso-enzymes (A and B) which can be distinguished by their electrophoretic mobility. Its synthesis in a cell is coded for by a particular gene on the X chromosome. In females only one of

the X chromosomes is active in each cell. The other one condenses during interphase as a Barr body. The process of inactivation occurs randomly in early development. Thus a heterogenous female with both A and B types of iso-enzyme will have 50 percent of cells coding for each enzyme. Unfortunately these individuals are rare but do provide a model for the study of clonal events. Fialkow et al. (1978) has observed that when female heterozygous for G-6-PD enzyme develop CML only one iso-enzyme is expressed in the erythrocytes, granulocytes,platelets, monocytes/macrophage and B lymphocytes in an individual patient. This suggests that all myeloid cells and B lymphocytes are members of a clone that developed from a single precursor. T lymphocytes were not derived from the same clone when tested during remission, but during the phase when the disease was poorly controlled T lymphocytes were derived from the malignant clone. Furthermore evidence for a common origin of stem cell for the myeloid and lymphoid lines was suggested by the work of Prachal et al. (1978). The same type of iso-enzyme of G-6-PD was shown to be present in all blood cells including T lymphocytes in a patient with acquired sideroblastsic anaemia while the skin fibroblasts and salivary gland cells contained the A and B mixed iso-enzymes.

1.1.7 Self-renewal capacity of CFU-S

The self-renewal capacity of CFU-S can be measured by the ability of cells from a spleen colony to produce secondary colonies when injected into secondary recipients (Siminovitch et al. 1963; Schofield et al. 1978). The number of colony forming cells in the colonies was markedly variable from one colony to another, with few colonies containing large numbers of CFU-S and most of the colonies containing very few CFU-S (Siminovitch et al. 1964 and Schofield et al. 1980). The mean CFU-S per colony of bone marrow derived from mice treated with cytotoxic agents was also changed. Schofield et al. (1980) showed that the spleen colonies derived from mice treated with continuous irradiation or the cytotoxic drug myeleran produced less secondary CFU-S compared to the colonies derived from normal bone marrow. Following serial transfer the mean CFU-S per colony was also decreased with increasing number of transfers.

The probability of self-renewal of CFU-S (p) also could be calculated from the data of mean CFU-S content of individual colonies and the standard deviation (Vogel et al. 1968). The value of (p) is here defined as the fraction of CFU-S which will produce another CFU-S during the growth of the CFU-S population in a colony, while (1-p) will be lost by differentiation or death during each generation cycle. However this value is not necessarily a measure of the physiological behaviour of CFU-S in-situ or in the experimental situations. It is only a measure of the potential capacity of CFU-S under standard conditions of elevated proliferation and differentiation pressures. The (p) value of CFU-S for normal bone marrow was found to be 0.68 which was higher than CFU-S from the bone marrow of irradiated mice (Schofield et al. 1980). On the other hand the average value of p for CFU-S from normal bone marrow determined theoretically by Vogel et al. (1968) and Lajtha et al. (1971) was lower than that reported by Schofield et al. (1980)

In the bone marrow the self-renewal probability of CFU-S however does depend on their location. Those in the centre of the marrow space were found to have higher self-renewal capacity than those located close to the bone surface (Lord and Schofield, 1980).

1.1.8 Age structure of CFU-S population

The CFU-S population was shown to have a heterogenous self-renewal capacity. Worton et al. (1969) fractionated bone marrow cells into various sub-populations by using the velocity sedimentation technique where the cells are separated on the basis of their size. In the slowly sedimenting cell fraction, the CFU-S population had a higher capacity to undergo self-renewal than the rapidly sedimenting CFU-S or the unfractionated population.

A dual population of CFU-S with regard to self-renewal was suggested by Schofield and Lajtha, (1973). The spleen colonies produced from bone marrow of donors two hours after isopropyl methane sulphonate (IMS) treatment were shown to be

virtually devoid of CFU-S whereas 70 percent of colonies from control donors contain at least 5 CFU-S. The IMS was suggested to selectively kill the self-maintaining CFU-S. The CFU-S which survived were a population with a limited proliferative potential and were lost from the CFU-S pool as a result of differentiation. An extreme highly self-renewal capacity of CFU-S could also be obtained from bone marrow mice treated with 5-fluorouracil (5-Fu) (Rosendaal et al. 1979). Rosendaal et al. (1976) also reported that the femoral CFU-S of mice injected with five doses of hydroxyurea was three times higher in secondary colonies than untreated mice.

Schofield (1978), Schofield et al. (1980) and Rosendaal et al. (1979) using CFU-S from different treatments and sources were able to show that different catagories of CFU-S existed from those with a very high self-renewal through a sequence of CFU-S with reducing capacity for self-renewal. Schofield (1978) and Rosendaal et al. (1979) postulated that CFU-S were organized with an age structure. The CFU-S population was suggested as a continuum of cells with changing capacity for self-renewal and differentiation. The younger CFU-S had a very high self-renewal capacity and were less susceptible to differentiation stimuli whereas the

the older CFU-S had a low self-renewal capacity and susceptible to differentiation stimuli (Schofield, 1979). Furthermore Monette and Stockel (1981) showed that the young CFU-S were not associated with a brain antigen whereas 85 to 90 percent of CFU-S population would be inactivated when incubated with anti-mouse brain serum.

1.1.9 The spleen colony forming cells and the haematopoietic stem cell

The number of CFU-S in the femoral marrow of old mouse was reported to be unchanged when compared to the young mouse and also to have the same repopulating ability when transplanted into irradiated recipients (Lajtha and Schofield, 1971). Harrison (1973) transplanted the bone marrow cells of old and young control mice into W/W^V recipients and the anaemic recipients were cured due to repopulation of donor cells. The effectiveness of bone marrow of old mice was shown to be the same as the young mice. Further transfers of marrow from old cured mice were carried out up to the fourth transfer and the result showed no sign of loss of repopulating ability.

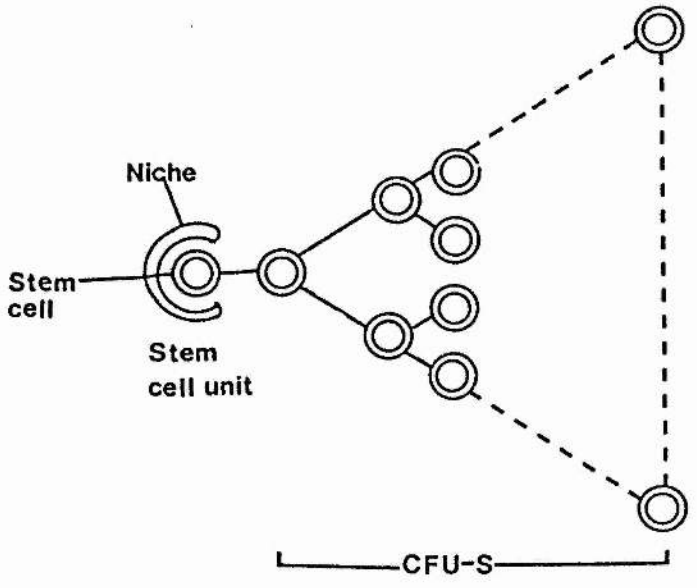

Figure 1.1.9: A representation of the haematopoietic stem cell unit (stem cell and niche) and its progeny, the spleen colony forming cells (Schofield, 1978).

Schofield (1974), calculated normal rate of turnover of CFU-S in normal mice which indicated that about 20 percent of the population were renewed each day. During the life span of 1000 days each of the CFU-S would complete about 200 doublings. In serial transfer of bone marrow from old donors to W/W^V mice, the stem cells had to expand in order to repopulate the recipient and to maintain their own population in each transfer. Therefore the CFU-S population had probably undergone more than replications and still did not show signs of exhaution of its replicating ability (Schofield, 1978).

A hypothesis was introduced by Schofield (1978) to provide an explanation of the age structure of the CFU-S population and the evidence that no differences in repopulating ability existed between CFU-S derived from young and old mice. He proposed that the CFU-S were not the ultimate haematopoietic stem cells. The true stem cells and the CFU-S were considered as two separate entities. Both are pluripotent and capable of self-renewal but the stem cell would have indefinite and the CFU-S limited capacity for self-renewal. The true stem cell exists in the marrow associated with other cells which provide a microenvironment

influence (a niche) and in isolation it would form a spleen colony.

Furthermore Schofield (1978) suggested that when the stem cell divided in the niche, one of the daughter cells would be retained in the niche while the other became a first generation of colony forming cells. The colony forming cells will then undergo a finite number of divisions and thus develop an age structure. Irradiation treatment damages some of the CFU-S and the niches. The niche is more radioresistant than CFU-S therefore vacancies are left available. The vacant niches can then be reoccupied by the CFU-S population with various self-renewal potential from endogenous or from injected bone marrow in the case of transplantation. As a result some of the niches could be refilled with CFU-S with lower proliferative capacity than the original stem cells.

In the serial transfer experiments, even though the bone marrow graft contained an equal number of CFU-S to those of the primary graft, the proportion of the CFU-S with high repopulating ability which can occupy the niches and become stem cells is smaller with each successive transfer in the control mice.

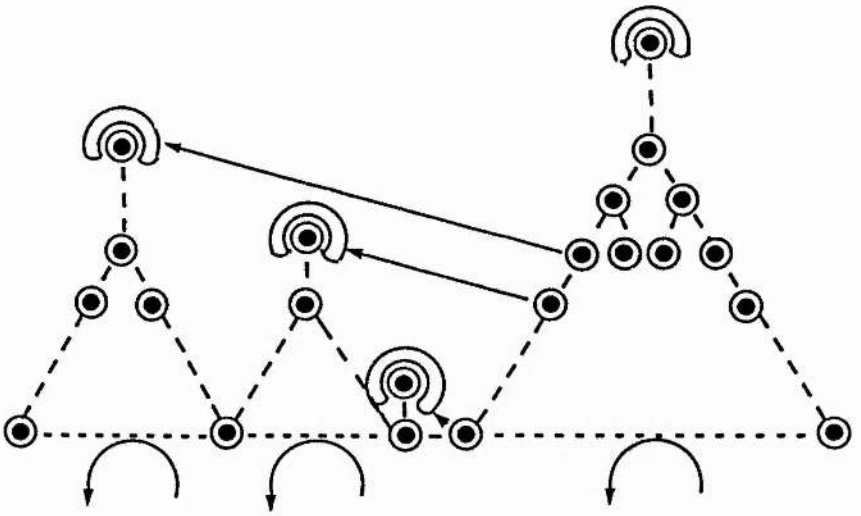

Figure 1.2.1: A postulate of the repopulation of depleted stem cell niches by spleen colony forming cells of different ages (Schofield, 1978).

1.2 Differentiation of haematopoietic stem cells

Differentiation of the stem cells is the process by which stem cells are removed from the stem cell population. A first step along differentiation pathways is to transform pluripotent cells into committed progenitor cells. Through further processes of differentiation and maturation the haematopoietic lineages were than established (Schofield and Lajtha, 1974).

Several committed progenitors of blood cells have been characterized by using in-vitro clonal assays. The first culture assays to be described were for the progenitors of the granulocytes and macrophages (Pluznik and Sachs, 1965 ; Bradley and Metcalf, 1966). Bone marrow or spleen cells were cultured in soft agar in the presence of colony stimulating activity (CSA). There are three distinct morphological types of colonies that were observed. The first type is a tight colony, which consists predominantly of granulocytes in varing stages of differentiation from myeloblasts to polymorphs. The second type is the loose colony, the cells in the colony are widely separated and usually consist of macrophages. The third type is a mixed

colony with a dense cellular centre with a corona of scattered, diffuse cells. The proportion of these colonies observed however is dependent on the source of the CSA used (Bol and Williams, 1980).

The committed progenitors of the erythroid cells were detected when haematopoietic cells were cultured in plasma clot (Axelrad et al. 1974) or methylcellulose (Stephenson et al. 1971) in the presence of erythropoietin. There were two types of erythroid colonies observed. The first was called colony forming unit-erythroid (CFU-E) which can be detected after a two day period of incubation. The sizes of colonies are small and contain up to 60 late normoblasts and nucleated erythrocytes. The second type of colonies is larger than CFU-E. These colonies is composed of several thousand cells which required 8 to 14 days of incubation for mouse bone marrow (Gregory and Eaves, 1978) and about 10 to 14 days for human cells (Ogawa et al. 1976). These large colonies were called burst colony forming units-erythroid (BFU-E). Although the presence of erythropoietin is essential for full differentiation of BFU-E, other factors, burst promoting activity (BPA), were also known to be required (Iscove, 1972). The BFU-E appeared to be an earlier erythroid precursor than the CFU-E.

1.2.1 Models of stem cell differentiation

From the analysis of spleen colonies, several models of stem cell differentiation have been postulated.

1.2.1.1 Haematopoiesis engendered at random (HER)

In this model, the determination of stem cell differentiation was considered to occur stochastically or randomly (Till et al. 1964). This model was postulated based on the analysis of the distribution of CFU-S, CFU-C, BFU-E and CFU-E in spleen colonies. The distribution of CFU-S in colonies was shown to be heterogenous, where the variance greatly exceeded the mean. This distribution would not fit a poisson distribution but conformed much more closely to a gamma distribution. The assumption was made that during the development of the spleen colony the probability of self-renewal and differentiation occurred in a random fashion. This assumption was further supported by a Monte Carlo calculation. The number of CFU-C, BFU-E and CFU-E were also reported to vary from colony to colony irrespective of colony morphology. These data suggested

that the individual cells within the population were not closely regulated but the population as a whole is regulated by varying the probabilities of self-renewal and determination for differentiation.

1.2.1.2 Haematopoietic inductive microenvironment (HIM)

In this model determination of the stem cell to differentiate occurs as a result of extra cellular inductive influences from the immediate microenvironment (Wolf and Trentin, 1968). This was postulated following studies of the histological composition of spleen colonies. The morphological properties of colonies in the spleen are dependent on the geographic position. The colonies found on the surface of the spleen are predominantly erythroid whereas those inside the spleen are mostly granulocytic. The ratio of erythroid and granulocytic colonies derived from the injection of bone marrow cells was less than one and for spleen cells was approximately 3.5. This ratio remained the same when the bone marrow or spleen was retransplanted into secondary recipients (Trentin, 1970). This model suggested that the direction of differentiation

of haematopoietic stem cells is controlled by their interaction with environment of the spleen or bone marrow stroma.

Furthermore it has been suggested that humoral factors produced in the bone marrow or spleen may influence the choice of CFU-S to differentiate towards erythropoiesis or granulopoiesis. The ratio of E/G colonies derived from bone marrow cells of mice treated with ARA-C increased significantly compared to those colonies derived from normal bone marrow (Frindel, 1979). A similar increase was also observed when bone marrow cells incubated with bone marrow of mice treated with ARA-C were transplanted (Frindel et al. 1980). The modification of the E/G ratio was assumed to be due to changes at the CFU-S level (Frindel and Vendrely, 1984).

1.2.1.2.1 Long term bone marrow culture (LTBMC)

In long term bone marrow culture the haematopoiesis was shown to be maintained for many months (Dexter et al. 1977). The establishment of adherent cells from bone marrow is vital in supporting the proliferation and differentiation of CFU-S. In the absence of adherent cell layer for example by using siliconized culture flasks to inhibit cell attachment, the sustainence of CFU-S was not achived. After inoculation of fresh bone marrow cells into adherent layers, the CFU-S that are present in the marrow inoculum migrate into the adherence layer and establish sites of active haematopoiesis (Dexter et al. 1978). As a result the committed progenitor granulocyte/macrophage cells (GM-CFC) (Dexter et al. 1978), primitive erythroid progenitor cells (BFU-E) (Eliason et al. 1979; Gregory and Eaves, 1978), megakaryocytes precursor (William et al. 1978) and CFU-S were shown continuosly present in the over lying growth medium. Cells with lymphoid potential were also being produced in this long term culture (Jones-villeneuve and Phillip, 1980).

The adherent cell layers produced from fetal liver, spleen and embryonic fibroblast however did not maintain the CFU-S proliferation and differentiation (Reimann and Burger, 1976). Furthermore the adherent layer established from Sl/Sl^d mice which have a defect in the environment but not in the CFU-S was defective in their capacity to support haematopoiesis in vitro (Dexter and Moore, 1977).

It was suggested that adherent layers in long term bone marrow culture reconstituted haematopoietic microenvironment (Dexter et al. 1977; Dexter et al. 1978). Three cell types were observed in the adherent layer: endothelial cells, phagocytic mononuclear and giant fat cells (Allen, 1981). Recently, Song and Quesenberry (1984) have shown that the adherent cells obtained from bone marrow of mice irradiated at 10 Gy were also capable of supporting CFU-S and GM-CFC population. They have shown that two type of cells were present in adherent layers. The first was a macrophage like cells and the second was epitheloid like cells.

1.2.1.2.2 The fibroblastic colony forming cells (FCFC)

The fibroblastic colonies were shown to be established when cell suspensions of bone marrow, spleen, lymph node and blood were cultured in vitro in the presence of feeder layer (Friedenstein, 1976). The cells in these colonies are characterized by their ability to produce collagen and morphological features such as cytoplasmic fibrils and distinctive nucleolar complexes (Friedenstein, 1976). From the study of tritiated thymidine labelling, chromosome markers, time lapse photography and linear dependence of colony number upon number of cells explanted, each of the colonies of fibroblast was shown to be clonally derived (Friedenstein et al. 1970;1974).

The fibroblast cells harvested with trypsin were shown capable of forming fibroblastic colonies when inoculated into fresh culture. Retransplantation fibroblastic cells derived from bone marrow in vivo in diffusion chambers were shown to form a calcified tissue in similiar manner to total bone marrow cells . On the other hand fibroblasts derived from spleen formed a reticular tissue (Friedenstein et al. 1974). Furthermore when fibroblastic colonies of bone

marrow were transferred under the kidney capsule, the development of bone and bone marrow was observed. On the other hand reticular tissue populated with lymphoid cells was observed when the fibroblasts are transplanted from spleen cultures (Friedenstein et al. 1974). Fibroblastic colony forming cells were suggested responsible for the transfer of the microenvironment typical of the given haematopoietic tissue (Friedenstein et al. 1974).

The progenitor cells of fibroblastic cells are more radioresistant than CFU-S or GM-CFC, and in the mouse the mean Do under X-rays or gamma rays is 2.23±0.23 Gy (Hendry and Lord, 1983). In mouse femur, a high concentration of FCFC was observed in the axial zone and decreased in the marginal zone (Xu and Hendry, 1981).

1.2.1.3 The quantal cell cycle

If cells were grown in-vitro in the presence of fetal calf serum and conditioned media from mitogen stimulated spleen cells, colonies containing erythrocytes, macrophages, neutrophils, eosinophils and megakaryocytes were observed

(Metcalf et al. 1978). Erythroid cells were always observed in association with other haematopoietic cells. Non-erythroid colonies were also detected in this culture system such as neutrophils, neutrophils and macrophages, macrophages, eosinophils and megakaryocytes. When these colonies were removed and cultured under similar conditions only mixed erythroid colonies were capable of producing further mixed colonies (Metcalf et al. 1978). Eosinophil colonies , neutrophil colonies, neutrophil and macrophage colonies or macrophage colonies were also capable of producing secondary colonies. Eosinophil colonies also could produce further eosinophil colonies but pure megakaryocyte and erythroid colonies could not produce secondary colonies of any kind (Johnson and Metcalf, 1978). From these observation Johnson (1981) proposed that multipotential cells are triggered to differentiate and a progressive loss of differentiation occurs at each stage

Lord (1983) suggested this observation was consistent to the Holtzer bipotential principle (Holtzer, 1978). Furthermore Lord (1983) refined this model in order to relate the evidence of an age structure of CFU-S compartment and the principle of a niche being occupied by a basic stem cells (see Figure 1.2.2). When stem cells in the niche

divide, one daughter remains in the niche as a new stem cell while the other cell has the potential to differentiate. At this stage it is capable of producing all the major haematopoietic cell lines. Following each division however it sequentially loses the capacity to differentiate along specific pathways. The pattern of loss differentiation capacity is summarized in figure 1.2.2. Evidence for this hypothesis has been described by Johnson (1981) following serial transfer of mixed colonies in-vitro.

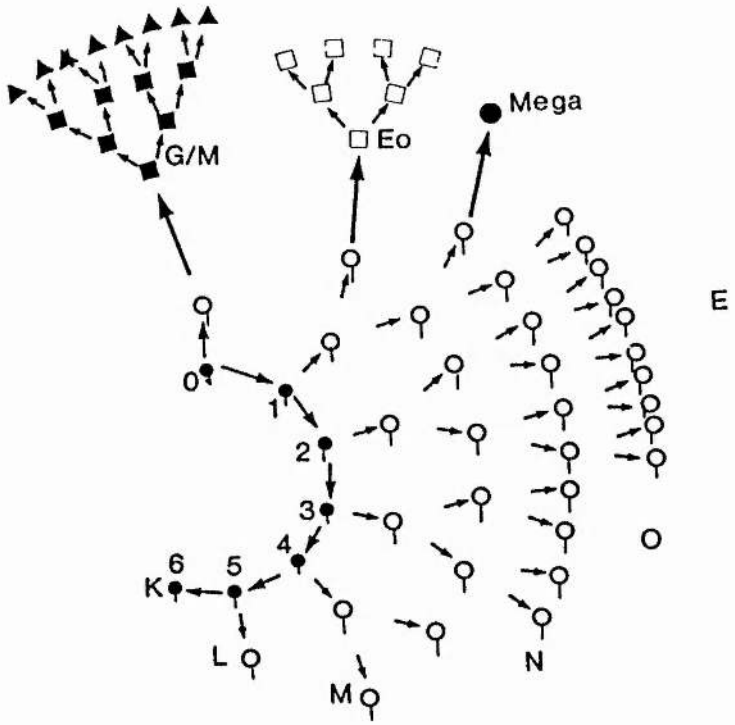

Figure 1.2.2: Generation of haematopoietic tissue from a single cell at position K_o, 'a niche position'. G - granulocyte, M - macrophage, E_o- eosinophil, Mega - megakaryocyte, E - erythrocyte, K → 0 - quanta stages, ♀ indicates a cell replaced by two cells in the quanta stage (Lord, 1983).

1.3 Regulation of CFU-S proliferation

1.3.1 Proliferative status of CFU-S

The proliferative status of CFU-S is normally determined by the suicide test. In this test the haematopoietic cells were incubated with tritiated thymidine (^{3}HTDR) (Becker et al. 1965; Lord et al. 1974) or cytosine arabinoside (ARA-C) (an S-phase cytotoxic drug) (Riches et al. 1981a). The numbers of colonies formed on the spleen were reduced compared to the control of haematopoietic cells incubated with medium, due the killing of CFU-S in DNA synthesis by ^{3}HTDR or ARA-C.

In the haematopoietic tissues of the normal adult most of the CFU-S population are in a non-proliferative state. The proportion of CFU-S synthesizing DNA was about 10 percent (Becker et al. 1965). During regeneration after treatment with radiation or cytotoxic drugs, the CFU-S population were found in a highly proliferative state. About 30 to 50 percent were in DNA synthesis (Lord et al.

1977). In fetal liver the CFU-S population was also actively synthesizing DNA, about 30 percent of CFU-S were killed by ^{3}HTDR or ARA-C (Becker et al. 1965; Thomas et al. 1981).

1.3.2 Local proliferative control

Proliferation of CFU-S in haematopoietic tissue may well be controlled at a local level. This suggestion came from the phenomena of differential proliferation of CFU-S in different tissues in the same animal (Croizat et al. 1970; Gidali and Lajtha, 1972; Rencricca et al. 1970 ; Wright and Lord, 1977). For example, in partial body irradiation experiments, the number of CFU-S in the shielded areas decreased considerably soon after exposure, the perentage of CFU-S in DNA synthesis increased markedly after initial depletion and returned to normal levels after that (Croizat et al. 1970; Gidali and Lajtha 1972). Mean while in the irradiated marrow where the CFU-S population was markedly depopulated, the CFU-S population still continued to cycle rapidly (Croizat et al. 1970 ; Gidali and Lajtha 1972).

The same phenomenon was also observed by Rencricca et al. (1970) and confirmed by Wright and Lord (1977) in mice treated with phenylhydrazine. The concentration of CFU-S in the bone marrow had fallen to about 50 percent whereas in the spleen the CFU-S had increased to about 3 to 4 times control values 7 days after treatment. The CFU-S population was proliferating maximally in the bone marrow but in the spleen at the same time the rate was the same as controls (Wright and Lord, 1977).

1.3.3 Humoral factors controlling CFU-S proliferation

CFU-S proliferation has been shown to be controlled by humoral factors. An inhibitory factor was shown to be present in haematopoietic tissues where CFU-S were proliferatively quiescent (Wright et al. 1977; Frindel et al. 1976). On the other hand a stimulatory factor was shown to be present in regenerating bone marrow or fetal liver (Lord et al. 1977; Becker et al. 1965; Thomas et al. 1981).

The evidence for the presence of inhibitory and stimulatory factors of CFU-S proliferation has been tested by groups of workers in Britain and France. The British group used supernatant free of cells (conditioned media) from haematopoietic tissues and tested for the presence of inhibitor or stimulator by incubating them with the non proliferative CFU-S population (normal bone marrow cells) or highly proliferative CFU-S population (regenerating bone marrow) (Lord et al. 1977; Wright et al. 1977; Cork et al. 1980; Riches et al. 1981a; Thomas et al. 1981). On the other hand the group in France used a system which is composed of two cellular compartments, based on a combination of organ culture and tissue culture (Frindel et al. 1976; Croizat and Frindel, 1980)

1.3.4 Inhibitor and stimulator of CFU-S proliferation

The inhibitor and stimulator in bone marrow extract can be concentrated into different molecular weight bands by using an an Amicon Diaflo ultra-filtration cell (Lord et al. 1976; Cork et al. 1981). The inhibitor was found to

concentrate only in fraction IV with molecular weight 50 to 100 K daltons from normal bone marrow extract and stimulator was found concentrated in fraction III with molecular weight 30 to 50 K daltons from regenerating bone marrow extract. It was also shown that inhibitor can be concentrated in fraction IV of bone marrow extract of rat, pig and human (Wright et al. 1981; Riches et al. 1981b). Frindel and Guigon (1977) both reported that inhibitor of CFU-S proliferation was also present in fetal calf bone marrow extract and can be detected in the dialysable fraction of the extract.

The effect of inhibitor and stimulator has now been shown to be specific to the CFU-S population. Lord et al. (1976) had showed that fraction IV of normal bone marrow extract had no effect on the proliferation of committed progenitor cells such as GM-CFC and CFU-E. Inhibitor was also shown to have no effect on the proliferation of PHA stimulated lymphocytes (Cork et al. 1980). Stimulator was also shown to be specific for the CFU-S population (Cork et al. 1982).

1.3.5 Characteristic of inhibitor and stimulator producing cells

From cell fractionation studies using a bovine serum albumin density gradient, Wright and Lord (1978) showed that inhibitor and stimulator were produced by cells in the lower density fractions. Inhibitor activity was produced by cells with a density of 1.052-1.060 g cm^{-3} and stimulator was produced by cells with a density of 1.064-1.072 g cm^{-3}. Whilst in the fraction which was enriched for the CFU-S population neither inhibitory nor stimulatory activities were detected. Producer cells were shown to have many of the properties of macrophages such as possessing the Fc receptor and being phagocytic. However not all macrophages are capable of producing the factor (Wright et al. 1981; 1982).

1.3.6 Mode of action of inhibitor and stimulator

The effect of inhibitor and stimulator was shown to be competitive when used in combination with each other in-vitro (Wright et al. 1977). These two factors are not mutually destructive since they can be incubated together for 25 hours or more and retain their activity after separation (Lord and Wright, 1982). Wright et al. (1977) and Lord et al. (1977) suggested that the level of CFU-S proliferation in an animal may be dependent on the balance of inhibitor and stimulator production. Although there was no inhibitory activity reported in RBME IV from regenerating marrow or no stimulatory activity reported in NBME III from normal quiescent marrow, these factors could be detected in concentrated sub-populations from density fractionated samples. Thus both inhibitor and stimulator producing cells are present in all populations but the balance of the factor production depends on the kinetics of the tissue.

1.3.7 The location of Go-CFU-S in cell cycle

The CFU-S population can be rapidly triggered into cell cycle by means of radiation (Lahiri and Van Putten, 1970), steroids (Byron, 1972) or cytotoxic drugs (Smith et al. 1968). This led to the suggestion that cells are held up in Go in late G1 or at the G1-S boundary. Furthermore, in series of experiments using various combinations of inhibitor (NBME IV) and stimulator (RBME III) together with vinblastine as a mitotic blocking agent, Lord (1980) showed that the location of Go was at the end of G1.

1.3.8 The action of inhibitor and stimulator

The action of inhibitor and stimulator on CFU-S proliferation seems to operate as a switch to on or off (Lord et al. 1979). Stimulator increased the proportion of CFU-S proliferating from normal bone marrow maximally after 30 minutes of incubation. On the other hand inhibitor had no measurable effect within this incubation time. It was suggested that inhibitor does not affect directly the CFU-S in DNA synthesis. From the time and dose response effect of inhibitor on CFU-S proliferation, Lord et al. (1979) showed for a period of 2 hours incubation a threshold dose of inhibitor is necessary before affecting the CFU-S proliferation. For the long period of incubation lower doses of inhibitor are required to have an effect on CFU-S proliferation. It was suggested that at the threshold dose, the inhibitor was effective only at the start of the G1 phase and this permits the G1 cells to progress normally into the S-phase. As the dose is increased, the effect is extended throughout the G1 phase to the G1-S boundary and eventually effectively blocking the entry of cells into S-phase and thus resulting in an earlier inhibition.

1.3.9 CFU-S proliferation in long term bone marrow culture (LTBMC)

In long term bone marrow cultures, CFU-S proliferation can be maintained for long periods of time (Dexter et al. 1977). This CFU-S proliferation was observed in both the adherent and non-adherent layers. When the cultures were depleted by removing half of the growth medium the CFU-S population in the adherent and non-adherent layers were highly proliferative, about 30 to 50 percent of CFU-S were in DNA synthesis. The proliferative activity however returned to normal levels one week after feeding (Dexter et al. 1978).

The inhibitory and stimulatory factors of CFU-S proliferation were shown to be present in the medium from LTBMC and can be concentrated in the same Amicon fractions as inhibitor in normal bone marrow extract or as stimulator in regenerating bone marrow extract (Toksoz et al. 1980). High levels of stimulator activity were shown in the culture medium shortly after feeding where CFU-S are actively cycling. When this culture medium was fractionated the stimulator was retained in fraction III. However in fraction IV a low level of inhibitor has been detected. On

the other hand in 7 days post feeding culture media high levels of inhibitory and low levels of stimulatory factors were detected.

Furthermore when the exogenous NBME IV inhibitor was added to newly fed culture the CFU-S proliferation in both the adherent and the non-adherent layer was reduced to normal level. When RBME III was added to cultures fed 8 days previously the proportion of CFU-S in DNA synthesis was increased to maximun 18 hours after addition. Thus it has been suggested that the proliferation of CFU-S in the culture system mimic the changes observed in-vivo (Toksoz et al. 1980).

1.4 Effect of radiation on haematopoietic stem cells (CFU-S)

Haematopoietic tissue is one of the most sensitive to radiation. Depletion of haematopoietic stem cells is one of the major factors which causes the failure in the supply of mature functional blood cells. This leads to the death of an animal after a few grays of whole body X-irradiation and is known as the bone marrow syndrome. The development of the spleen colony assay (Till and McCulloch, 1961) for measuring the reproductive intergrity of stem cells has enabled quantitative studies to be made after exposure to radiation.

1.4.1 CFU-S survival curve

The survival curve was first measured by Till and McCulloch (1961). The survival fraction of CFU-S was usually obtained by comparing the number of CFU-S in the femur or spleen after irradiation to the number of CFU-S in the femur or spleen of unirradiated mice. The survival can be measured only up to a dose of 4.0 to 6.0 Gy, about the third

decade of depopulation. For doses above this range, it was difficult to measure due to the limiting size of the inoculum to ensure a significant number of colonies (Hendry and Lord, 1983).

The dose which was required to inactivate or reduce survival to 37 percent is known as D_{37} or Do. The second parameter from the survival curve was the extrapolation number (n) which is obtained by extrapolating the straight line of the survival curve to the survival fraction axis. The Do of bone marrow CFU-S after gamma irradiation in-vitro was 1.05 Gy and the n value was 2.5 (Till and McCulloch, 1961). However when irradiation was performed in-vivo the D_{37} was slighty decreased but the extrapolation number (n) decreased to about 40 percent (McCulloch and Till, 1962). In other studies, Hendry (1972) reported that there were no significant differences the in D_{37} or n values after the in-vivo or the in-vitro irradiation either with gamma rays or X-rays or 14 MeV neutrons.

The sensitivity of CFU-S populations from different sources was shown to vary. Siminovitch et al. (1965) showed that CFU-S derived from fetal liver were more radioresistant than CFU-S derived from bone marrow or

spleen. However 14 days after transplantation into lethally irradiated recipients, the sensitivity was close to control. Hendry (1972) also reported that the sensitivity of CFU-S obtained from femoral bone marrow of mice 7 days following 4.5 Gy whole body X-irradiation was lower compared to CFU-S from normal bone marrow. However the observation that cycling CFU-S were less radiosensitive was not always consistent. Duplan and Feinendegen (1970) showed that CFU-S surviving thymidine suicide were less radiosensitive as compared to CFU-S in S-phase.

1.4.2 Effect of different LET

Radiations with different linear energy transfer (LET) were known to produce different biological effects. The response of CFU-S to different LET radiations were investigated by Hendry (1972) and Carsten and Bond (1976). Hendry (1972) showed that the D_{37} for neutrons of CFU-S from femoral marrow, regenerating bone marrow and spleen were lower than for X-rays and gamma rays. The extrapolation number of neutrons for femoral CFU-S was also shown to be lower than X-rays and gamma rays but no significant

differences were observed for splenic and regenerating bone marrow CFU-S. However in a study using monoenergetic neutrons produced by an accelerator, the radiosensitivity of CFU-S bone marrow was shown increase with increasing energy (Carsten and Bond, 1976).

The relative biological effectiveness (RBE) of monoenergetic neutrons (the ratio of D_{37} of X-rays to the D_{37} of neutrons) was about 2.7 for the range of energies between 0.43 to 1.8 MeV (Carsten and Bond, 1976). However neutrons with 13.4 MeV the RBE value was less than unity (Carsten and Bond, 1976). The RBE for the fission neutrons of reactor or ^{252}CF was shown to be lower than for the monoenergetic fast neutrons.

The RBE of neutrons for CFU-S derived from different sources was shown to be different (Hendry, 1972). The sensitivity of CFU-S to low LET radiation (X-rays and gamma rays) was also reported to be different (Hendry, 1972).

1.4.3 The effect of bone

The absorbed dose of radiation by the marrow during exposure is directly influenced by the bone which encloses the marrow cavities. This influence is due to differences in physical properties of bone and marrow. During exposure to 200 - 300 kVp X-rays, the energy absorbed by soft tissue close to bone was higher than that absorbed by soft tissue remote from bone surfaces. The emission of secondary electrons from the bone surface leads to the adjacent soft tissue receiving a higher dose than expected (Spiers, 1949).

Epp et al. (1959) by using calculations developed by Spiers (1949)have shown that for 250 kVp X-rays the effect of secondary electrons is significant up to 200 μm from the bone surface whereas gamma rays of ^{60}Co produce very few secondary electrons. Lord and Hendry (1972) showed that the secondary electrons would increase the dose to the femoral marrow by 6 percent. This minor effect can be detected when axial and marginal CFU-S are assayed separately after a specified dose of 200 kVp X-rays. The ratio of numbers of CFU-S in the marginal and axial zones of normal unirradiated

mice is 1.69. This ratio was uneffected by 1.2 Gy of gamma rays, but is reduced to 1.32 by 1.0 Gy of 200 kVp X-rays. In the vetebrae of the mouse where the marrow is in the form of a lattice of spheres of 200 μm in diameter with 80 μm of bone wall thickness, the enhancement of the dose would be about 12 percent compared to the gamma rays. The ratio of the bone surface to volume in vetebrae was higher compared to the femur. As a consequence, the percentage of CFU-S killed in vetebrae was about 40 percent higher than in the femur (Lord, 1978).

The effect of bone on the absorbed dose of marrow during fast neutrons exposure however was contrary to the effect of X-rays (see Figure 1.4.3). It was shown that the dose would be lower when close to the bone surface after 14 MeV neutrons exposure (Yammamoto, 1966). The effect on the CFU-S population can be shown by measuring the ratio of CFU-S surviving in the femur and vetebrae (see table 1.4.3).

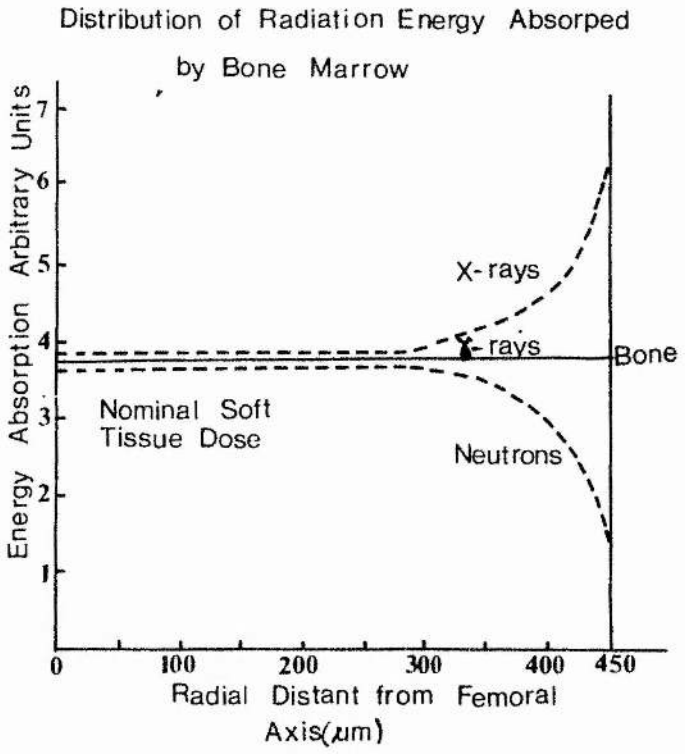

Figure 1.4.3: Diagramatic illustration of the radiation energy absorbed by bone marrow following irradiation by X-rays, gamma rays and neutrons.

Table 1.4.3: Effect of radiation quality and the presence of bone on the spatial distribution of CFU-S.

	Marginal CFU-S	Femoral CFU-S survival
	Axial CFU-S	Vetebral CFU-S survival
Control	1.69 ± 0.17	1.0
1.0 Gy X-rays (200 kVp)	1.32 ± 0.15	1.37 ± 0.15
1.2 Gy γ-rays (^{137}Cs)	1.68 ± 0.14	1.10 ± 0.11
2.0 Gy neutrons (14 MeV d+T)	-	0.74 ± 0.11
	(Lord and Hendry, 1972)	(Lord, 1978)

1.4.4 Effect of dose rate

Krebs and Jones showed the D_o and n values of CFU-S were changed when irradiated at dose rate of 0.00286 Gy min^{-1} to 1.05 Gy min^{-1}. However Glassgow et al. (1973) reported there was no change in sensitivity of CFU-S to radiation when this range of dose rates was used. At the very low dose rate, 0.5 Gy day^{-1}, Porteous and Lajtha (1966) reported the survival curve of CFU-S was changed to a biphasic curve. The initial phase of the curve is much steepler than the second phase. The slope of the initial curve was shown not to be dose dependent and also not significantly different from the curve of acute single dose (Wu and Lajtha, 1975). The slope of the initial curve was suggested to represent the sensitivity of quiescent CFU-S. As the accumulation of the dose increased above 3.0 Gy or about 2 percent of CFU-S survival, then the second phase of the curve was observed and was dependent on the dose rate. On the other hand, by using the same low dose rate reported by Wu and Lajtha (1975), Kalina et al. (1975) showed that the D_o was higher than for an acute single dose and increased with decreasing dose rate.

1.4.5 Post irradiation dip

After initial depletion following whole body gamma or X irradiation, the number of CFU-S in the femur and spleen was further decreased from few hours to 48 hours (Guzman and Lajtha, 1970; Hendry and Howard, 1971; Hendry, 1972). This decrease was called the post irradiation dip. The dip however was not dependent on the dose. In the femur between the doses of 0.5 Gy to 9.0 Gy the dip was to about 55 percent whereas in the spleen this loss is more severe, about 90 percent between 1.0 Gy to 5.5 Gy (Hendry and Lord, 1983). A similar dip was also observed when unirradiated CFU-S were grafted into a primary recipient (Lahiri et al. 1970).

The dip can be abolished by various treatments given before irradiation such as endotoxin (Smith et al. 1966) and cyclophosphamide (Millar et al. 1978). The dip was shown to reduce when mice were exposed to high LET radiation (Hendry and Howard, 1971).

1.4.6 Regeneration of CFU-S population

After the post irradiation dip, the CFU-S population begins to regenerate by increasing the proportion of CFU-S entering into the cell cycle. The rate of the recovery however was dependent on the initial depletion after irradiation. At low doses the recovery rate is slower and takes alonger time to reach normal levels as compared to higher dose (Chervinick and Boggs, 1971).

At a dose above 3.0 Gy the initial depletion of CFU-S is greater than 1.5 decades, the mean doubling time of CFU-S in the femur was about 28 hours and 16 hours for CFU-S in spleen. Recovery to the normal values occurs within 3 to 5 weeks. There was no evidence of an over shoot either in spleen or femur. The doubling time of CFU-S in the femur was similar to that of normal CFU-S grafted into lethally irradiated recipients. It was suggested that this value represents the optimal rate for repopulation and differentiation of CFU-S in haematopoietic tissue. Schofield and Dexter (1982) reported that after 0.5 Gy of whole body X-irradiation, CFU-S do not rapidly repopulate their number to normal level. An initial depletion of CFU-S

in the femur to about 60 percent occurred followed by a further decrease to 30-40 percent 24 hours later. However, 25 days later the CFU-S had recovered only to 70 percent of control CFU-S numbers and plateaued at 50 percent level after 30 to 55 days. The CFU-S however did finally reach the normal levels after two months.

1.4.7 Residual effect of CFU-S

The CFU-S population both in the femur and spleen has been shown to recover to normal level after a single sub-lethal dose (Coggle, 1980). However there are also several reports showing that the CFU-S numbers were unable to recover to normal level (Carsten and Bond, 1979; Covelli and Metalli, 1973; Croizat et al. 1979). The concentration of the CFU-S per mm femur shaft length was also reported to decrease to about 1/3 of unirradiated control after 7.0 Gy of X-rays (Covelli and Metalli, 1973). Furthermore Croizat et al. (1979) reported that the cycling rate of CFU-S was increased 6 months after exposure to 5.0 Gy of 4.5 MeV electrons.

The residual damage of CFU-S however was more obvious when repeated irradiation was performed allowing time for repopulation between doses (Hendry and Lajtha, 1972). The CFU-S population in the femur and spleen recovered only to sub-optimal levels. In the femur the recovery was about 20 to 30 percent of control 24 days after the fourth dose and this was maintained for up to one year. This sub-optimal recovery was even lower when irradiation was performed with neutrons (Hendry et al. 1974). In spite of this plateau the CFU-S population was actively cycling as fast as normal regeneration. The rate of CFU-S recovery after the fourth dose was slower and the doubling time was longer compared to the recovery after a single dose of 4.5 Gy X-rays. However when grafted into lethally irradiated recipients the doubling time was similar to unirradiated CFU-S but the CFU-S from the repeated 4.5 Gy dose animals failed to colonize the femoral marrow of the recipients to the same level as unirradiated CFU-S.

MATERIALS AND METHODS

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CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

Female and male CD1 mice were used for most of the experiments. Female CBA/H and C57BL/10ScSn/Ola mice were used for some experiments where specifics H-2 types were required. CD1 mice were supplied by Charles River U.K and bred in the University of St.Andrews animal house. CBA/H mice were supplied by the M.R.C. Radiobiological Institute, Harwell and C57BL/10ScSn/Ola mice were supplied by Olac 1976 Ltd., Oxfordshire and both were maintained as inbred stocks in St.Andrews.

Mice were kept in a room with a light cycle of 12 hours light and 12 hours dark at a temperature of 22°C. Mice were fed with R&M No.1 pellets (BP., Whitham, Essex) and water containing 15 ppm of free chloride ion (Kirbychlor, Mildenhall, Suffolk). Mice were used for the experiments at

an age of 8 to 12 weeks. During the experiment groups of 5 mice were kept in presterilized cages with saw dust covering the floor and kept inside a laminar flow cabinet. Saw dust was replaced every 3 or 4 day. The condition of the mice was checked everyday. Mice which appeared to develop infection were then killed.

2.2 Irradiation treatment

Irradiation treatment was performed by using a Siemens Stabilipan x-ray machine (250 KVP, 14 mA and 0.5 mm Cu filtration) in conjunction with an applicator which gave 20 x 20 cm^2 of radiation field. Farmer dosimeter type 2502/3 (Nuclear Enterprise Ltd.) with a thimble type ionization chamber was used to measure the dose rate. The chamber was placed in the centre of a wax mouse as a tissue equivalent phantom. The mean dose rate at different positions in the irradiation cage was taken. At 55.5 cm focalskin distance the dose rate was 0.88 Gy min^{-1}. Mice were irradiated in groups 10 in a perspex box, with individual radially disposed compartments.

2.2.1 In-vitro Irradiation

A plug of bone marrow was removed by flushing one ml of air into bone cavity with a one ml syringe and 23G x $1^{1}/4$ inch needle. The bone marrow plugs were placed on a millipore filter and irradiated on stainless steel grid in a petri dish containing medium. The dose rate was measured at the position where bone marrow plugs were irradiated. However TLD (thermoluminescent dosimeter) should be used such as LiF in order to get an accurate dose to the bone marrow plugs.

2.3 Haemopoietic cell suspension

Mice were killed by exposing to diethyl ether. The skin was swabbed with 70 percent alchohol before dissecting the tissues out.

2.3.1 Bone marrow cell suspension

The femur was dissected free of muscle and removed by cutting above the hip joint with scissors and disarticulated at the knee joint with a scalpel. The adherent muscles were stripped off and the femur was cut below the lesser trochanter. The femurs were kept in a petri dish containing cold Fischer's medium (Flow Laboratories Ltd., Scotland). The marrow cells were removed by inserting a 23G x $\frac{1}{4}$ inch needle into the inter condylar notch and flushed with one ml of medium through the bone cavity by using a one ml tuberculin syringe. To produce a single cell supension the medium was then drawn in and out of a 25G x $\frac{5}{8}$ inch needle.

2.3.2 Spleen cell suspension

The loose skin of the left side of the abdomen was cut and the peritoneum was then exposed by pulling the cut skin toward the head and the tail. The spleen was removed from the cavity by using forceps and scissors. The spleen was

teased gently in a petri dish containing 5 ml medium with forceps and scalpel. The suspension was drawn in and out of a 5 ml syringe attached to 23G x 1 inch needle, followed by a 23G x $1\frac{1}{4}$ inch needle. The cell suspension was then separated from the clumps of debris by shaking the suspension and allowing the clumps to settle for 5 minutes before transferring the suspension into another tube.

2.3.3 Normal peritoneal cell suspension

Skin covering the abdomen was incised and pulled toward the head and the tail. By using a syringe attached to 23G x 1 inch needle, 5 ml of medium were injected into the peritoneal cavity. The peritoneum was massaged for 3 minutes and then 3 or 4 ml of fluid from the cavity were then withdrawn. The fluid was discarded if the needle punctured the gut. The cells were pooled and washed once at 1000 rpm for 10 minutes (Chilspin Fisson).

2.3.4 Thymus cell suspension

The thorax cage was opened and the thymus lobes were removed by using fine forceps and scalpel and kept in a petri dish containing cold medium. The thymus lobes were cleaned from adherent connective tissue. By using fine forceps, the thymus was teased into small fragments. A single cell suspension was then obtained by using a syringe and 23G x 1 inch needle followed by a 23G x 1¼ needle. The suspension was then allowed to settle for 5 minutes then separated from the clumps of cells.

2.4 Cell count

A haemocytometer chamber was used to count cells in suspensions of spleen, thymus, peritoneal or sheep red blood cells. The suspensions were diluted to give 5-10 x 10^5 cells ml^{-1} and a minimum of 200 cells were counted.

Bone marrow cells were counted by using a Coulter Counter Model D (Industrial) (Coulter Electronic Ltd., England) using the following settings : Apparture = 4 mA, Amplification = 2 and Threshold = 20. Cells in suspension were counted by resuspending 40 ul of suspension into 20 ml of Isoton (Coulter Electronic Ltd., England) and 3 drops of Zaponin (Coulter Electronic Ltd., England) were added for lysing the red blood cells.

2.5 Cell viability

The viability of cells following preparation was determined by the trypan blue dye exclusion (Mishell et al. 1980) and acridine orange-ethidium bromide methods (Parks et al. 1979).

2.5.1 Trypan blue dye exclusion

One part of cell suspension at a concentration of 2 - 5 x 10^6 cells ml^{-1} was added to one part of trypan blue stock solution. The stock solution of trypan blue was prepared by mixing 4 parts of 0.2 percent trypan blue (w/v) in water with one part of 4.25 percent saline (w/v). The cell suspension was agitated gently and kept on ice. The cells were counted within 3 minutes by using a Haemocytometer chamber. Unstained cells were scored as viable cells and stained cells as dead cells.

2.5.2 Acridine orange-ethidium bromide method

Stock solution of acridine orange was prepared by dissolving 15 mg acridine orange and 50 mg ethidium bromide in one ml of 95 percent ethanol. The solution was then added into 49 ml distilled water. After the solution was mixed aliquots of one ml were kept at -20°C. During the experiment, one ml stock aliquots were diluted (1:100) in

phosphate buffered saline. One part of this solution was then mixed with one part of cell suspension at a concentration of 10^6 cells ml^{-1}. The number of cells fluorescing green and orange were counted by using a haemocytometer chamber and fluorescence microscope. Cells fluorescing green were scored as viable cells and cells fluorescing orange were scored as dead cells.

2.6 Supernatants prepared from bone marrow (conditioned media)

Bone marrow cells were suspended at a concentration of 5×10^7 cells ml^{-1} in Fischer's medium. Suspension was centrifuged at 1000 rpm for 10 minutes. Supernatant free of cells (conditioned media) was then spun at 3000 rpm for 15 minutes to remove any further debris. Conditioned media were then sterilized by passing supernatant through a millipore filter of diameter 0.2 μm and stored at $-20^{\circ}C$ before being used.

2.7 Fractionation of conditioned media

Bone marrow cell suspensions of mice 7 days following 4.5 Gy whole body X-irradiation were prepared in cold saline. The cell concentration was adjusted to $5 \times 10^6 - 1 \times 10^7$ cells ml^{-1} and then kept at $4^{\circ}C$ on ice. The first supernatant was obtained by centrifugation at 1000 rpm for 10 minutes. The pellets of cells were then resuspended in equal volumes of saline and further incubated at $37^{\circ}C$ for 3 hours A second supernatant was then obtained by centrifugation by the same procedure as the first supernatant. The supernatants were pooled as the crude irradiated bone marrow extract (CBME). Any debris was removed by spinning at 4000 rpm for 20 minutes. The CBME was then fractionated by using ultra filtration with Amicon Diaflo filter membranes (Diaflo Ultra Filter, Lexington Massachusetts) (Lord et al. 1976). Fraction III with molecular weight 30 - 50 K daltons and fraction IV with molecular weight of 50 - 100 K daltons were retained and freeze dried.

2.8 Spleen colony assay

Spleen colony assay was performed according to the method of Till and McCulloch (1961). Recipient mice were exposed to 8.2 Gy of whole body X-irradiation. At this dose level no endogenous colonies were observed. Mice were warmed by using a photographic tray heater and a 60 watts table lamp for about half an hour to dilate the tail veins. Bone marrow cells in a volume of 0.2 to 0.5 ml were intravenously injected into the tail vein by using one ml syringe and 25G x $\frac{5}{8}$ inch needle. The dose of cells was adjusted to produce 10 to 15 colonies per spleen. For normal bone marrow 4×10^4 cells were injected. After 8 days mice were killed and spleens were biopsied and fixed in Bouin's solution for 24 hours. Colonies were counted immediately after fixation or after transfer to 70 percent alchohol for 24 hours before counting. A dissecting microscope was used for counting at X20 magnification.

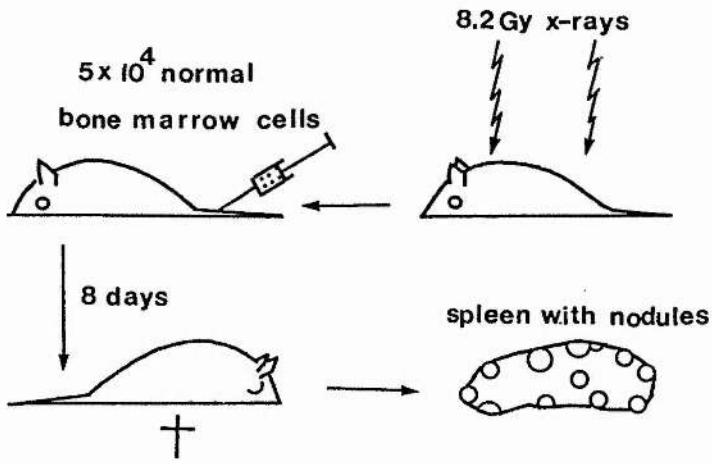

Figure 2.8: Technique for the production of haematopoietic clonal nodules on the spleens of 8.2 Gy whole body X-irradiated mice by injection of syngeneic bone marrow cells.

2.9 Measuring the percentage of CFU-S population in DNA synthesis

The percentage of the CFU-S population undergoing DNA synthesis was measured by using a suicide technique. An S-phase specific drug cytosine arabinoside (ARA-C) (UpJohn) was used (Riches et al. 1981a).

Bone marrow cell suspensions in Fischer's medium containing 10 percent of horse serum (Flow Laboratories Ltd., Scotland) were prepared at a concentration of 2×10^6 - 1×10^7 cells ml^{-1}. Duplicate of one ml aliquots were pipetted into 10 ml polysterene tubes. Into one of the tubes, 100 µl of ARA-C (20 µg) was added while the same volume of medium was added into the control tube. The suspensions were then incubated in a water bath at a temperature of $37^{o}C$ for 60 minutes. After incubation, cells suspensions were transferred to an ice bath and diluted with cold medium. The CFU-S numbers were then assayed in groups of 10 to 15 irradiated recipients.

The percentage of CFU-S in DNA synthesis was then calculated by comparing the number of colonies in the control sample with colonies in the sample treated with ARA-C. The standard error of percentage of CFU-S killed by ARA-C were calculated by using the formula used by Lord et al. (1974).

$$K = \frac{(C - T)}{C} \times 100$$

Where; C = mean colonies in the control sample

T = mean colonies in the ARA-C treated sample

$$SE_k = \frac{100}{C}\sqrt{SE_t^2 + (T/C)^2 \times SE_c^2}$$

Where; SE_c = standard error of colonies in the control sample

SE_t = standard error of colonies in the ARA-C treated sample

SE_k = standard error of percentage of CFU-S killed by ARA-C

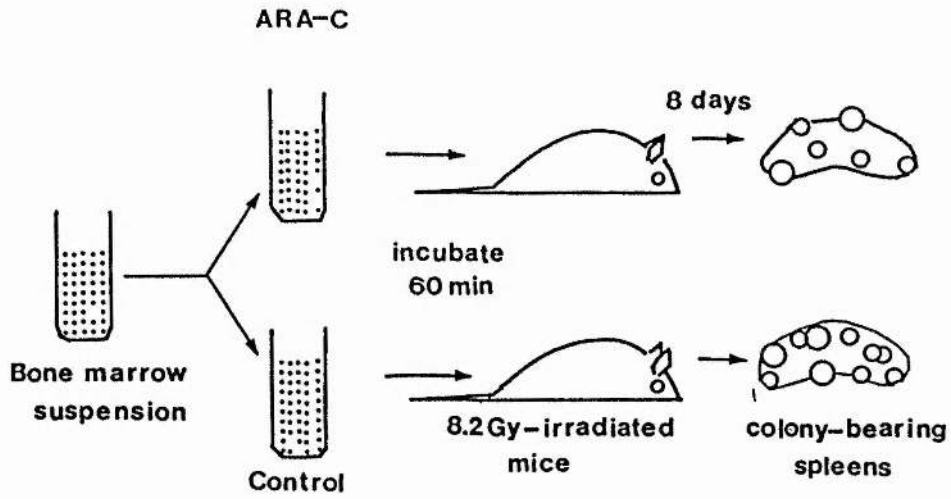

Figure 2.9: ARA-C suicide technique for the measurment of the proportion of CFU-S in a bone marrow cell suspension which is synthesizing DNA.

2.10 Assay of conditioned media (CM) and fraction III

The presence of CFU-S proliferation stimulators in the CM and fraction III was assayed according to the protocol described by Lord et al. (1978). Normal bone marrow cells were used as a source of minimally proliferating assay CFU-S.

The bone marrow cells of irradiated mice were suspended at a concentration of 2 - 5 x 10^6 cells ml^{-1} and prepared for the production of conditioned media (see section 2.6). The CM were assayed on the basis of the cell number used in the preparation : e.g 0.5 ml of assay cells containing 2 x 10^6 ml^{-1} mixed with 0.5 ml CM prepared from a suspension containing 6 x 10^6 cells ml^{-1} gave a ratio of 1:3 (assay/CM) in cell number. This has been defined as a 75 percent concentration of CM (Wright and Lord, 1979).

Duplicates of 0.5 ml of normal bone marrow suspension at a concentration of 2 x 10^6 cells ml^{-1} were mixed with CM. For the fraction III, a concentration of 60 µg ml^{-1} mixed with the cells. The mixtures were then incubated at 37°C

for 2 hours and the percentage of CFU-S population undergoing DNA synthesis was determined (see section 2.9).

2.11 Assay for granulocyte-macrophage progenitor cells (GM-CFC)

Granulocyte-macrophage colony forming cells were assayed in semi solid agar with the presence of colony stimulating factor (CSF) obtained from the mouse myelomonocytic leukemia WEHI-3 cell line conditioned media.

Bone marrow cells were suspended in the RPMI 1640 medium (Flow Laboratories, Scotland) supplemented with 20 percent horse serum, 2 mM Glutamine 50 i.u ml^{-1} penicillin and 50 mg ml^{-1} Streptomycin. The cell concentration was adjusted to a concentration of 1 x 10^6 cells ml^{-1}. 3 percent of Bacto-agar (Difco) was boiled for about 10 minutes before being used for the experiment. One ml of boiled agar was added into 8 ml of medium and followed by the addition of one ml cell suspension. The mixture was then thoroughly mixed and aliquots of one ml were immediately dispensed into 35 mm petri dishes (Sterilin

Ltd., Middlesex) containing 100 µl of WEHI-3 conditioned media. The cultures were allowed to gel for 10 minutes and incubated at 37°C in an atmosphere of air containing 10 percent CO_2. Colonies consisting of more than 50 cells were counted after 7 days of incubation by using an inverted microscope at X40 magnification.

2.12 Cell separation

2.12.1 Plastic adherence separation

Cells were separated on the basis of their differential adherence properties to plastic petri dishes. A cell suspension in Fischer's medium at a concentration of 5×10^6 cells ml^{-1} was prepared. Aliquots of 2 ml were pipetted into 35 mm diameter petri dishes. Cells were allowed to adhere to petri dishes in an atmosphere of 5 percent CO_2 in air for 2 hours at 37°C. Non-adherent cells in the medium were removed by using a pasteur pipette and the petri dishes

were washed twice by adding fresh medium. The adherent cells were removed by scraping them off with a rubber policeman. The cells were washed twice by centrifugation (1000 rpm , 10 minutes) and resuspended in Fischer's medium.

2.12.2 Bovine serum albumin (BSA) cell separation

Discontinuous BSA gradients were used to fractionate bone marrow cell populations (Wright et al. 1981;1982). This procedure was used to separate rosettes.

2.12.2.1 Unbuffered balance salt solution (UBSS)

The UBSS was used for preparing the bovine albumin solution at mouse osmolarity (308 mOsmolar ; equivalent to 0.168 M NaCl). It was prepared by mixing the following solutions : 121 parts of 0.168 M NaCl, 4 parts of 0.168 M KCl, 3 parts of 0.112 M $CaCl_2$, 1 part of 0.168 M $MgSO_4.7H_2O$ and one part of 0.168 M KH_2PO_4. The solution was then

sterilized by passing through a membrane filter (0.45 μm).

2.12.2.2 Preparation of BSA

BSA (Fraction V, Sigma Laboratories Ltd., England) was dissolved in distilled water to make a 15-20 percent (w/v) solution. This solution was then dialysed against deionized distilled water for 48 hours at 4°C. Deionized distilled water was changed every 6 hours and the contents of the dialysis bag were thoroughly mixed. Pre-cooled water was used and several drops of chloroform were added to each change of water to prevent bacterial growth (Shortman, 1968). After dialysis the albumin solution was passed through a membrane filter (0.45 μm) and freeze dried. Albumin was then dried for 2 days in a vacuum dessicator over P_2O_5 powder to remove residual water.

2.12.2.3 Preparation of dense BSA stock solution

BSA stock solution at 35 percent (w/v) was prepared by dissolving 100 gm of dialysed dry albumin in 182 ml of UBSS and 4 ml of H_2O. The dry albumin was layered in small amounts onto 100 ml of salt solution in a sealed container (to prevent evaporation). The solution was kept at 4°C and stirred occasionally. The remaining salt solution was used to remove albumin from the flask walls. The albumin was dissolved after 48 hours. The stock solution was then stored at -20°C before being used.

2.12.2.4 Determination of the densities of the BSA solution

The densitiy of the solution was then calculated from the formula : density = 1.543u - 1.0553 (Leif and Vinograd,1964) where u = refractive index. The refractive index was determined by using a refractometer (Bellingham and Stanley Ltd.).

2.12.2.5 Preparation of discontinuous BSA gradients

The amount of BSA stock solution required to make up a known volume of a specific density was estimated by using the following formula .

$$X = \frac{(A \times B) - B}{C - 1}$$

Where; X = volume of BSA stock (ml)

A = desired density

B = volume of final solution

C = density of stock BSA solution

The final density of the solution was checked by using the refractometer.

A discontinuous BSA gradient was prepared in a plastic tube by using a bent Pasteur pipette. A volume of 0.3 ml BSA with a density of 1.10 gm cm^{-3} was used as a cushion at the bottom of the tube (figure 2.12.5). Cells were suspended in 2 ml of Fischer's medium and gently layered onto the top of the discontinuous gradients. The tube was then spun at 4000 rpm for 10 minutes (Chilspin Fisson) and the interface populations were recovered and washed 3 times and resuspended in Fischer's medium.

2.12 Fc-rosetting

2.12.1 Sheep red blood cells (SRBC)

SRBC were obtained from St.Andrews slaughter house and kept in Alsever's solution at a concentration of 50 percent (w/v). The buffy coat layer was then removed and SRBC were washed 3 times by centrifugation at 2000 rpm for 10 minutes with Alsever's solution and stored at $4^{o}C$ before use. For

the experiments, SRBC were washed 3 times with PBS by centrifugation (2000 rpm, 10 minutes) and resuspended at 10 percent (v/v) in PBS.

2.12.2 Preparation of 10 percent (SRBC) and 5 percent RBC-antibody

An equal volume of 10 percent SRBC suspension was mixed with SRBC antiserum (Nordic Immunological Laboratories, England) used at 1:80 dilution. The mixture was incubated at 37°C for 30 minutes (Parish and Haward, 1974). After incubation the mixture was centrifuged at 2000 rpm for 10 minutes and washed once with PBS. The SRBC coated with antibody were then resuspended to 5 percent (v/v) with PBS.

2.12.3 Preparation of Fc-rosette

Cell suspensions of bone marrow, spleen or peritoneal exudate were prepared at cell concentration of 1.5 - 2 x 10^7 cells ml^{-1}. Equal volumes of cell suspension and 5 percent of SRBC-precoated were rotated on a vertical rotator at 30 rev min^{-1} at 37°C in an incubator (Parish and Haward,

1974).

2.12.4 Counting rosettes

The mixture of cells and 5 percent of SRBC precoated antibody suspension were kept on ice and an aliquot of 0.1 ml was added to 0.4 ml PBS. One drop of 0.5 percent crystal violet was added and mixed gently. After 5 minutes the numbers of white cells and rosettes were counted by using a haemocytometer chamber. A rosette was scored as a single white cell attached to 5 or more sheep erythrocytes.

2.12.5 Separation of rosettes

The rosettes were separated from single cells by using BSA discontinuous gradients. Aliquots of 0.5 ml suspension were layered onto BSA density gradients (1.03 g cm^{-3} and 1.09 g cm^{-3}) (figure 2.15.5). The tube was centrifuged at 4000 rpm for 10 minutes. The interface populations were collected and the presence of rosettes was checked. The interface

population of 1.09 g cm^{-3} (more than 85 percent are rosettes) was suspended in one ml of tris-buffered ammonium chloride to lyse the red blood cells (section 2.12.6) and washed twice with medium and resuspended in Fischer's medium.

2.12.6 Procedure for lysing red blood cells

The pellet of cells was suspended with one ml tris-ammonium chloride solution (9 parts of ammonium chloride 0.16 M plus 1 part of 0.17 M tris (hydroxy methyl amino methane) adjusted to pH 7.2 with 1 M HCl (Hudson and Hay, 1976). After 7 minutes 10 ml cold medium were added to stop the treatment. Cells were spun and washed twice.

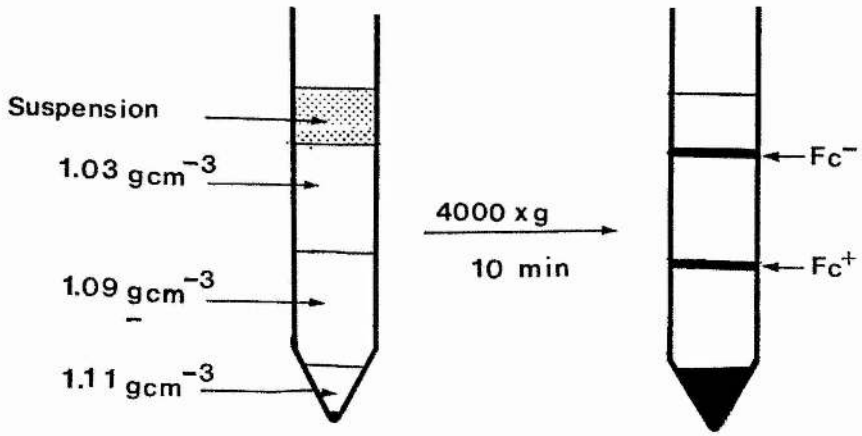

Figure 2.12.5: Separation of rosettes with discontinous BSA gradients.

2.13 Complement mediated cytolysis

Complement dependent cytotoxicity was used to deplete Thy1.2$^+$ and Ia-2K$^+$ cells. Cells carrying these antigens were killed with the aid of complement by directing antisera against cell surface antigens. The percentage of cells killed was determined by trypan blue or acridine ethidium bromide methods. The viable cells were separated using discontinuous BSA gradients (1.03 g cm^{-3} and 1.10 g cm^{-3}) (Wright et al. 1981).

2.13.1 Thy 1.2 cytolysis

0.5 ml of cell suspension at a concentration 2 x 10^7 cells ml^{-1} was mixed with 0.5 ml anti Thy 1.2 (Miles Laboratories (1:5 dilution) (Miles Laboratories Ltd.). Preliminary experiments with thymocytes showed that the maximal cell killing could be achieved with this antiserum concentration. The mixture of cells and anti Thy 1.2 was incubated at 4^{o}C for 30 minutes. Supernatant was removed after centrifugation (800 rpm, 10 minutes) and the pellet of

cells was resuspended in one ml of guinea pig complement (1:10 dilution) (Wellcome Reagents Ltd., England) and incubated for 45 minutes. After incubation cells were washed once and separated on the BSA gradients and finally washed 3 times before being suspended in Fischer's medium.

2.13.2 Ia-2K cytolysis

Cells were suspended in RPMI 1640 medium (Flow Laboratories Ltd., Scotland) with 25 mM Hepes buffer and 0.3 percent bovine serum albumin. The red blood cells were first lysed by incubating the pellet of cells with one ml tris ammonium chloride for 7 minutes. The cells were then washed twice with cold medium then resuspended at a concentration of of 2 x 10^7 cells ml^{-1}. An aliquot of 500 µl monoclonal anti Ia-2k (1:20 dilution) (Cedarlane Laboratories Ltd., Canada) was added to 500 µl of cell suspension. (Preliminary experiments showed that 1:40 dilution of monoclonal anti Ia-2k could kill 40-50 percent of CBA/H spleen cells). The mixture was then incubated at $4^{o}C$ for 60 minutes. After incubation the supernatant was discarded and cells were resuspended in one ml low toxicity

rabbit complement (1:6 dilution) (Cedarlane Laboratories Ltd., Canada) and further incubation was performed at 37°C for 60 minutes. Cells were washed twice and resuspended in Fischer's medium.

2.14 Direct immunofluorescence staining of Thy 1.2 cells

Cells were suspended in Minimum Essential Medium (MEM) containing 5 percent fetal calf serum, 10 mM Hepes and 0.1 percent sodium azide (Wofsy et al. 1980). The cell concentration was adjusted to 2 x 10^6 cells ml^{-1} and aliquots of 0.5 ml were centrifuged at 300 rpm for 7 minutes. Aliquots of 100 ul monoclonal anti-mouse Thy 1.2 fluorescein labelled (New England Nuclear, Boston, Massachusetts) (1:100 dilution) were added to the cells pellet and suspended. The suspension was then incubated on ice for 30 minutes. After incubation one ml of fetal calf serum was added into cell suspension and mixed thoroughly. The suspension was centrifuged at 800 rpm for 10 minutes and the pellet of cells was suspended in MEM-FCS medium. The cells were washed twice with medium by centrifugation at 300

rpm for 7 minutes.

The pellet of the stained cells was resuspended in a small volume of 100 percent fetal calf serum to give a concentration of about 1-2 x 10^7 cellml^{-1}.

One small drop 10 ul of aliquot was placed on a slide and covered with a cover slip, the edge of cover slip was sealed with permount. Cells were examined at x500 magnification by using Zeiss fluorescence microscope with UV light source, BG3 exiter filter and 53/44 barrier filters. Cells in aggregate were discounted.

2.15 Statistical analysis

The significant test was calculated by using the following formula (Riches, 1985 personal communication) : $R = \frac{\text{different of means}}{\sqrt{se1^2 + se2^2}}$

where:

se1 = standard error of group 1

se2 = standard error of group 2

The significance points were then determined from the following table:

P	0.1	0.05	0.01	0.001
R	1.64	1.96	2.58	3.29

RESULTS

CONTENTS

CHAPTER 3

RESULTS

3.1 Changes in nucleated cell numbers and CFU-S numbers per femur following sub-lethal doses of whole body X-irradiation

Experimental procedure

Female CD1 mice were irradiated at the following doses: 0.50 Gy, 0.75 Gy, 1.50 Gy and 4.50 Gy of whole body X-irradiation. A dose rate of 0.63 Gy min^{-1} was used for the exposures of 0.50 Gy and 0.75 Gy. For the doses of 1.50 Gy and 4.50 Gy a dose rate of 0.88 Gy min^{-1} was used. The nucleated cell number and the CFU-S number in the femur were assayed at the following times: 30 minutes, 2 hours, 4 hours and 6 hours.

Results.

The number of nucleated cells in the femoral marrow of unirradiated CD1 mouse was $14.5 \pm 1.2 \times 10^6$ (table 3.1a) and $14.05 \pm 1.2 \times 10^6$ cells (table 3.1c and d). Following 0.50 Gy whole body X-irradiation the nucleated cell number was not significantly affected (figure 3.1.1). At sub-lethal doses of 1.50 Gy the number was reduced to 93 ± 9 percent at 30 minutes but increased to 123 ± 20 percent after 2 hours and fell to 88 ± 10 percent at 6 hours. Following 4.50 Gy, the nucleated cells were reduced to 82 ± 13 percent of control levels at 30 minutes and further decreased to 24 ± 13 at 6 hours (figure 3.1.1).

The total number of CFU-S in the femoral marrow of normal female CD1 mice was 4110 ± 450 (table 3.1d) and 4214 ± 602 (table 3.1c). Following 0.50 Gy the number was reduced to 94 ± 17 percent of control ($P>0.1$) when assayed at 30 minutes and 95 ± 22 percent of control ($P>0.1$) when assayed 6 hours after exposure (table 3.1a). At 0.75 Gy the number of CFU-S per femur was reduced to 37 ± 12 percent at 30 minutes and 28 ± 12 percent at 6 hours. After 1.50 Gy the

CFU-S number was reduced to 26±5 percent at 30 minutes, 33±9 percent at 2 hours and 23±5 percent at 6 hours. A further decrease of CFU-S per femur was observed when mice were exposed to a 4.50 Gy dose. The number of CFU-S in the femur fell to 0.32±10 - 0.34±11 percent of controls (figure 3.1.2).

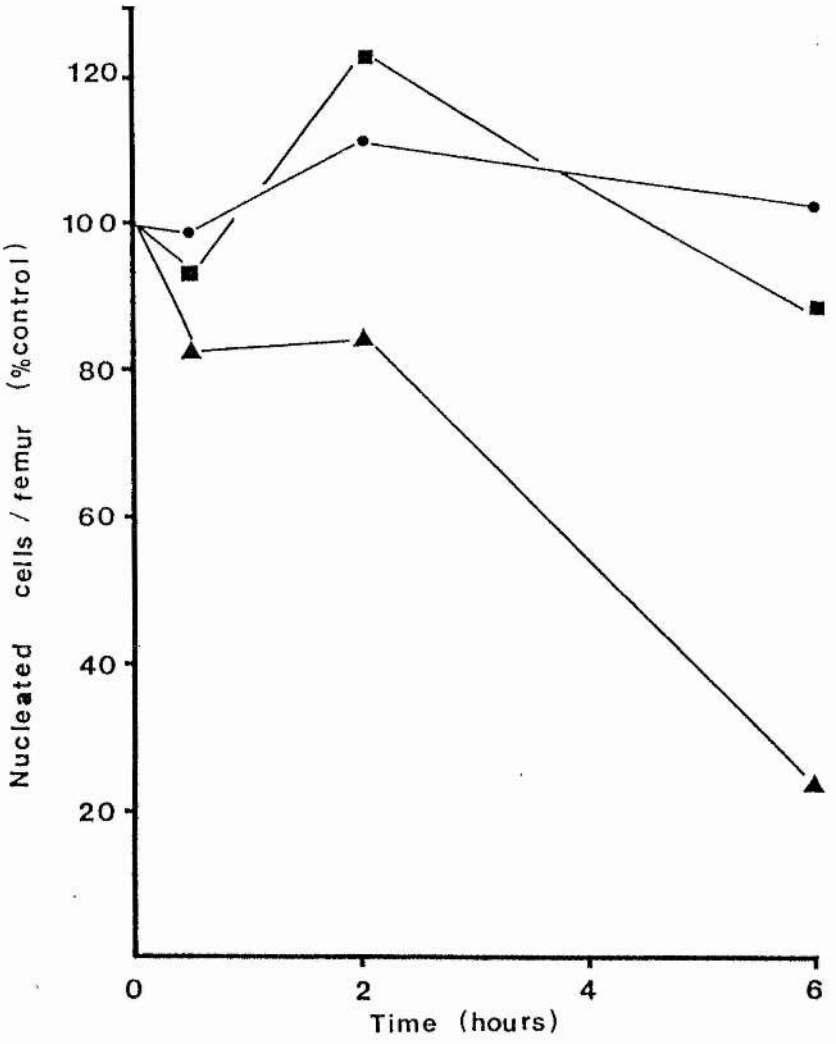

Figure 3.1.1. The percentage of nucleated cells per femur (mean of single experiments) within 6 hours following 0.50 Gy (●——●), 1.50 Gy (■——■), and 4.50 Gy (▲——▲).

Note - the femurs were prepared by removing the proximal epiphysis and thus results refer to numbers of nucleated cells per femoral shaft.

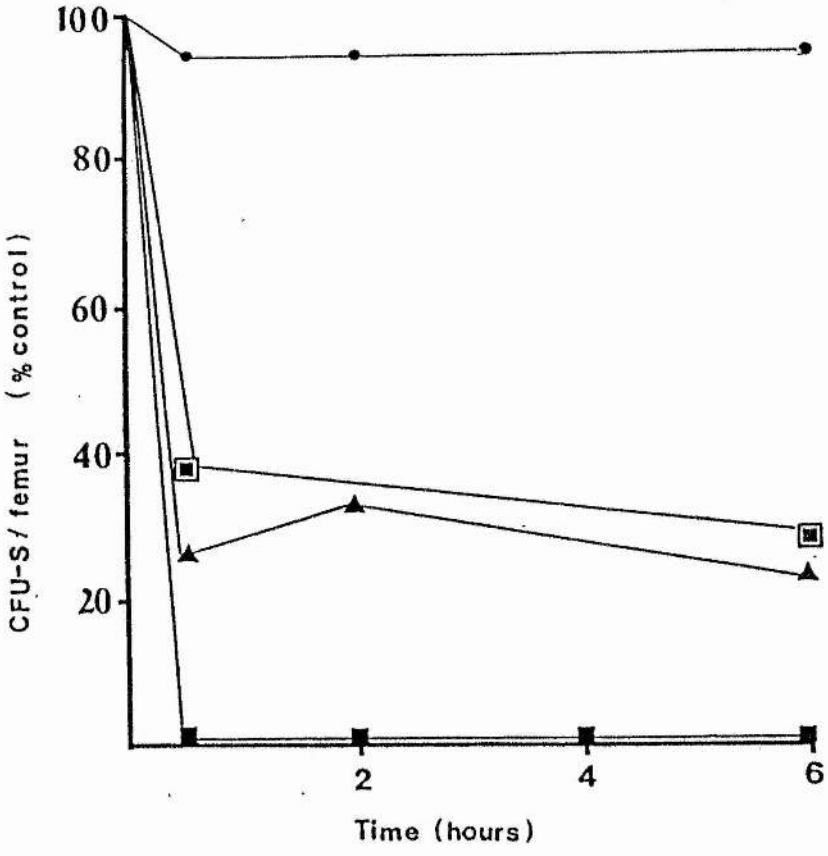

Figure 3.1.2. The percentage of CFU-S per femur (mean of single experiments) within 6 hours following 0.5 Gy (●——●), 0.75 Gy (▣——▣), 1.50 Gy (▲——▲) and 4.50 Gy (■——■).

Note - the femurs were prepared by removing the proximal ephiphysis and thus results refer to numbers of CFU-S per femoral shaft.

Table 3.1a: The number of nucleated cells and CFU-S per femur (mean ±SE of single experiments) within 6 hours following 0.50 Gy whole body X-irradiaton.

Time after irradiation (hours)	Nucleated cells per femur($x10^{-6}$)	% of control	CFU-S per femur	% of control
Control	14.5±1.2	100±12	4212±602	100±14
0.5	13.8±2.5	98±20	3979±355	94±17
2	15.6±0.4	111±9	3974±848	94±24
6	14.4±1.3	102±13	4020±677	95±22

Table 3.1b: The number of CFU-S per femur (mean ±SE of single experiments) within 6 hours following 0.75 Gy of whole body X-irradiation.

Time after irradiation (hours)	CFU-S per femur	% of control
Control	4212±602	100±14
0.50	1540±140	37±12
6	1140±226	28±12

Table 3.1c. The number of nucleated cells and CFU-S per femur (mean ±SE of single experiments) within 6 hours following 1.5 Gy of whole body X-irradiation.

Time after irradiation (hours)	Nucleated cells per femur($x10^{-6}$)	% of control	CFU-S per femur	% of control
Control	14.05±1.2	100±12	4212±602	100±14
0.5	13.11±0.1	93.3±9	1101±131	26±5
2	17.33±2.6	123.3±20	1386±381	33±9
6	12.40±0.6	88.0±10	967±148	23±5

Table 3.1d. The numbers of nucleated cells per femur and CFU-S per femur (mean ±SE of single experiments) within 6 hours of 4.50 Gy whole body X-irradiation.

Time after irradiation (hours)	Nucleated cells per femur($x10^{-6}$)	% of control	CFU-S per femur	% of control
Control	14.05±1.2	100±9.4	4110±450	100±11
0.5	13.60±2	81.9±13	13±4	0.32±10
2	14.0±2	84.3±13	14.1±1	0.34±11
6	4.0±2	24.0±13	14.0±1	0.34±11

3.2 The effect of low doses of whole body X-irradiation on CFU-S numbers in the femoral marrow.

Experimental procedure.

Groups of 10 female CD1 mice were irradiated at the following doses: 0.06 Gy, 0.10 Gy and 0.50 Gy whole body X-irradiation at a dose rate of 0.63 Gy min^{-1}. The CFU-S in the femoral marrow was assayed at one and two weeks after exposure.

Results.

Figure 3.2 shows that the percentage of CFU-S per femur after 0.06 Gy at one week was 91 ± 24 percent of control unirradiated mice ($P>0.1$). For the mice irradiated at 0.10 Gy and 0.50 Gy the numbers of CFU-S per femur assayed one week after were 81 ± 15 ($P>0.1$) and 66.3 ± 15 ($P=0.05$) percent of control unirradiated mice.

The number of CFU-S per 10^5 cells injected of normal bone marrow was 28.4 ± 4.0 and one week after 0.06 Gy it was 33.0 ± 5.9 ($P>0.1$) and after 0.10 Gy it was 38.1 ± 1.9 ($0.05>P>0.01$).

The numbers of CFU-S assayed in the second week after doses 0.06 Gy, 0.10 Gy and 0.50 Gy were not significantly different from unirradiated mice ($P>0.1$) (table 3.2b).

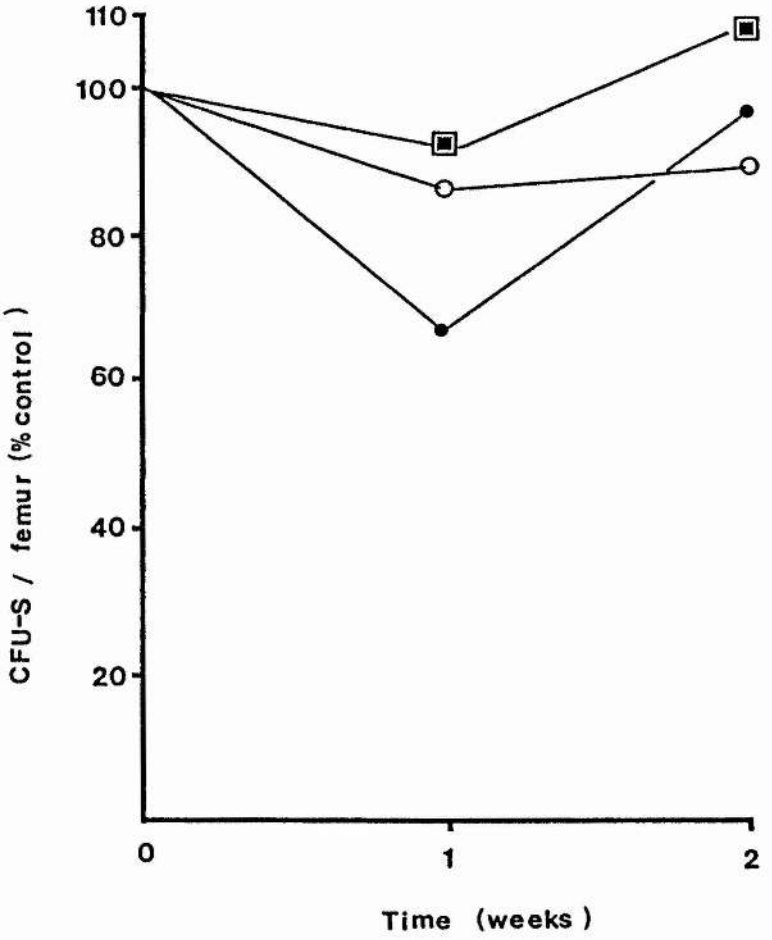

Figure 3.2: The percentage of CFU-S per femur (mean±SE of single experiments) at one and two weeks after low doses of whole body X-irradiation (▣——▣ 0.06 Gy, ○——○ 0.10 Gy and ●——● 0.50 Gy)

Table 3.2a and b : The number of CFU-S per 10^5 cells injected and CFU-S per femur (mean±SE of single experiments) at one and two week after 0.06 Gy, 0.10 Gy and 0.50 Gy of whole body X-irradiation.

(a) Week one

Dose (Gy)	CFU-S/10^5 cells injected	CFU-S per femur (% of control)
Control	28.4±4.0	100±14
0.06	33.0±4.0	91±24
0.10	38.1±1.9	81±15
0.50	19.1±1.7	66.3±15

(b) Week two

Dose (Gy)	CFU-S/10^5 cells injected	CFU-S per femur (% of control)
Control	27.0±2.1	100±9
0.06	28.0±2.9	107±23
0.10	24.1±3.5	89±13
0.50	25.5±2.9	96±13

3.3 The effect of 0.50 Gy whole body X-irradiation on Granulocyte -macrophage colony forming cells (GM CFC) in the femoral marrow.

Experimental procedure.

Groups of 5 CD1 mice were irradiated at 0.50 Gy dose (0.63 Gy min^{-1} dose rate). The GM-CFC in the femoral marrow were assayed by using the in-vitro semi solid agar culture system with the presence of WEHI-3 conditioned media (see materials and methods section 2.11). Normal bone marrow cells were used as control.

Results.

Figure 3.3.1 shows that following 0.50 Gy whole body X-irradiation the number of GM-CFC per femur was reduced to 14.2 ± 1.6 - 28.6 ± 4.7 percent of control levels between 30 minutes to 6 hours.

The number of colonies per 10^5 cells plated of normal bone marrow was 104.3±4.5, consisting of 57.7 percent of tight colonies, 31 percent of loose colonies and 11.3 percent of mixed colonies (table 3.3). The number of colonies per 10^5 cells plated of bone marrow following 0.50 Gy was between 14.8±1.1 to 29.8±1.5 (table 3.3). The proportion of colony type was also found to change, 80 percent of the colonies were loose type and 20 percent were tight colonies (figure 3. 3.2).

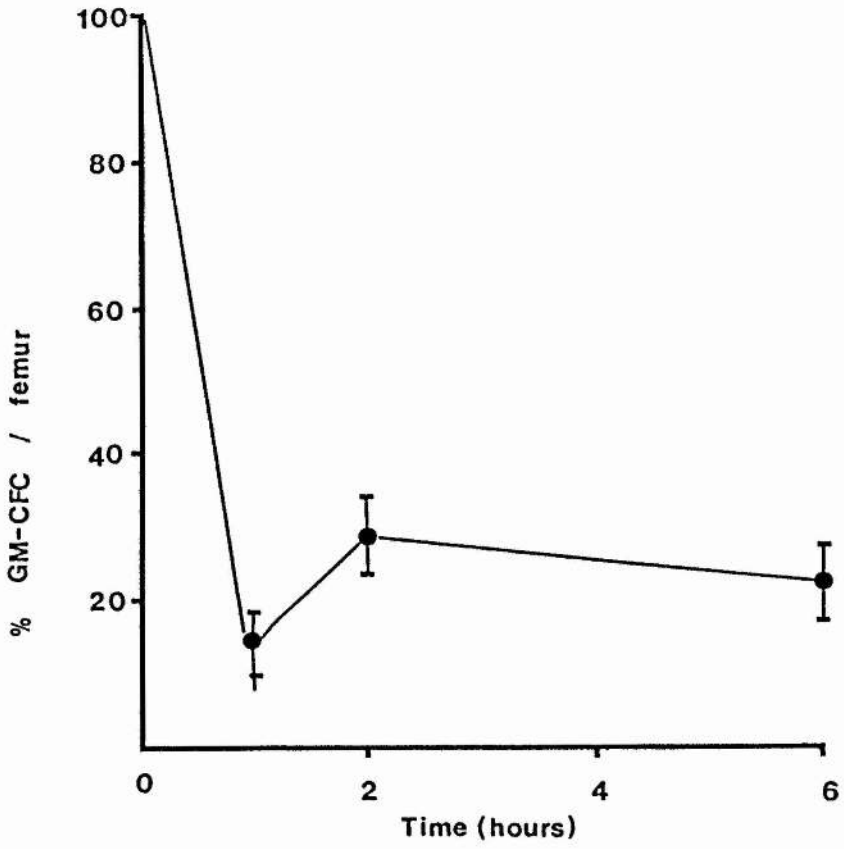

Figure 3.3.1: The percentage of GM-CFC per femur (mean±SE of single experiments) following 0.50 Gy whole body X-irradiation.

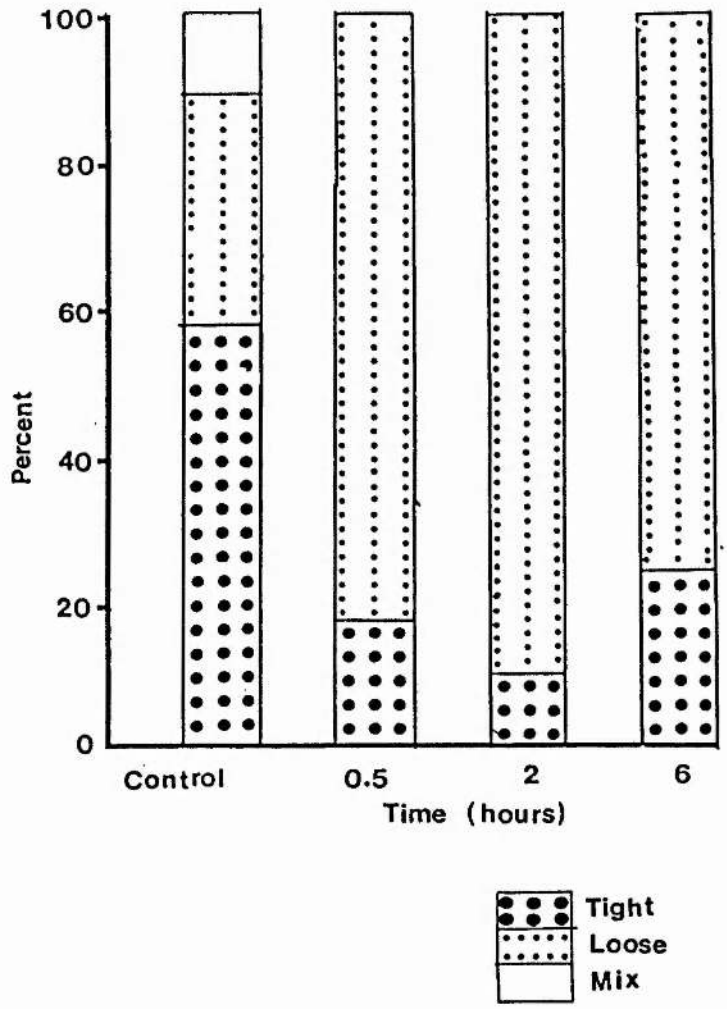

Figure 3.3.2: The proportion of different types of colonies per 10^5 cells plated after 0.50 Gy whole body X-irradiation between 30 minutes to 6 hours.

Table 3.3: The number of colonies per 10^5 cells plated and colonies types (mean±SE) within 6 hours following 0.50 Gy whole body X-irradiation.

Time after irradiation (hours)	Colonies per 10^5 cells plated	Types of colonies		
		tight	loose	mixed
Control	104.1±4.5	60.2±3.0	32.3±2.6	11.8±2.0
0.5	14.8±1.1	2.7±0.5	12.1±1.1	-
2	29.8±1.5	3.0±0.7	26.7±1.3	-
6	24.1±1.5	5.8±1.2	17.9±1.6	

3.4 Changes in proportion of CFU-S in DNA synthesis following low and sub-lethal doses of whole body X-irradiation.

Experimental procedure.

3.4.1 Determination of time of CFU-S entering into DNA synthesis following 0.50 Gy and 4.50 Gy doses of whole body X-irradiation.

Female CD1 mice were irradiated at 0.50 Gy (0.63 Gy min^{-1} dose rate) and 4.50 Gy (0.88 Gy min^{-1} dose rate) of whole body X-irradiation. The percentage of CFU-S in DNA synthesis was measured at the following times: 0.50 hours, 2 hours, 4 hours and 6 hours.

3.4.2 The percentage of CFU-S from the femoral marrow of CD1 and CBA/H mice in DNA synthesis after 0.50 Gy and 1.50 Gy doses.

Female CD1 mice and male CBA/H mice were irradiated at 0.50 Gy (0.63 Gy min^{-1} dose rate) and 1.50 Gy (0.88 Gy min^{-1} dose rate). The percentage of CFU-S in DNA synthesis was measured at 30 minutes after exposure.

3.4.3 The dose response curve of CFU-S entering into DNA synthesis at 2 hours and 6 hours after whole body X-irradiation.

Female CD1 mice were irradiated at the following doses: 0.50 Gy, 0.75 Gy, 1.50 Gy and 4.50 Gy and the percentage of CFU-S in DNA synthesis measured at 2 hours and 6 hours after exposure.

Table 3.4a: The percentage of femoral CFU-S in DNA synthesis (mean±SE of single experiments) within 6 hours following 0.50 Gy of whole body X-irradiation.

Time after irradiation (hours)	CFU-S per 10^5 cells injected	% CFU-S in DNA synthesis
Control	34.6±2.8	10.3±2.2
0.5	25.0±3.0	37.0±7.0
2	26.2±2.0	43.9±11.2
4	24.0±2.0	23.3±9.9
6	22.4±2.6	14.8±8.1

Table 3.4b: The percentage of femoral CFU-S in DNA synthesis (mean±SE of single experiments) within 6 hours following 4.50 Gy of whole body X-irradiation.

Time after irradiation (hours)	CFU-S per 10^6 cells injected	% CFU-S in DNA synthesis
Control	154.0±16.0	15.4±2.1
0.5	6.5±2.0	12.5±10
2	7.0±0.5	6.0±11
4	7.0±1.0	2.0±18
6	7.0±0.5	34.0±14

Table 3.4c : The percentage of femoral CFU-S in DNA synthesis (mean±SE of single experiments) of CD1 mice and CBA/H mice 30 minutes after 0.50 Gy whole body X-irradiation.

Mouse Strain	CFU-S per 10^5 cells injected		% CFU-S in DNA synthesis
	+ medium	+ ARA-C	
CD1			
Control	34.3±2.8	30.2±3.0	12.4±1.3
0.50 Gy	25.4±1.8	16.0±1.4	37.0±7.1
CBA/H			
Control	22.7±2.0	20.8±3.9	8.4±14.8
0.50 Gy	22.7±5.8	10.6±1.8	53.3±26.7

Table 3.4d: The percentage of femoral CFU-S in DNA synthesis (mean±SE of single experiments) of CD1 mice and CBA/H mice 30 minutes after 1.50 Gy whole body X-irradiation.

Mouse strain	CFU-S per 10^5 cells injected		% CFU-S in DNA synthesis
	+ medium	+ ARA-C	
CD1			
Control	242±14	224±18	7.4±9.2
1.50 Gy	34.0±3.8	34.7±3.2	-2.1±14.9
CBA/H			
Control	176±18	148±10	15.9±10.3
1.50 Gy	43.7±4.9	35.2±5.7	19.5±15.9

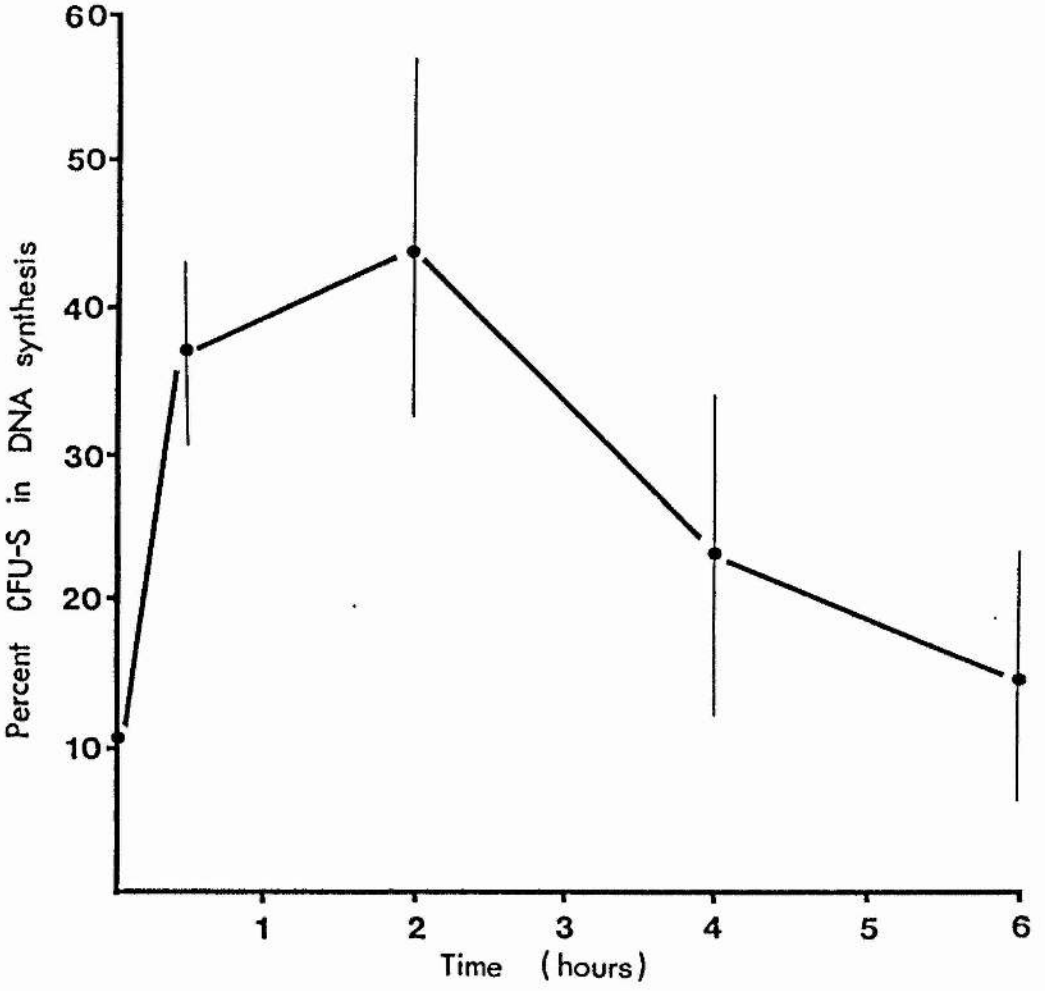

Figure 3.4.1: Percentage of femoral CFU-S in DNA synthesis (mean±SE of single experiments) following 0.50 Gy of whole body X-irradiation.

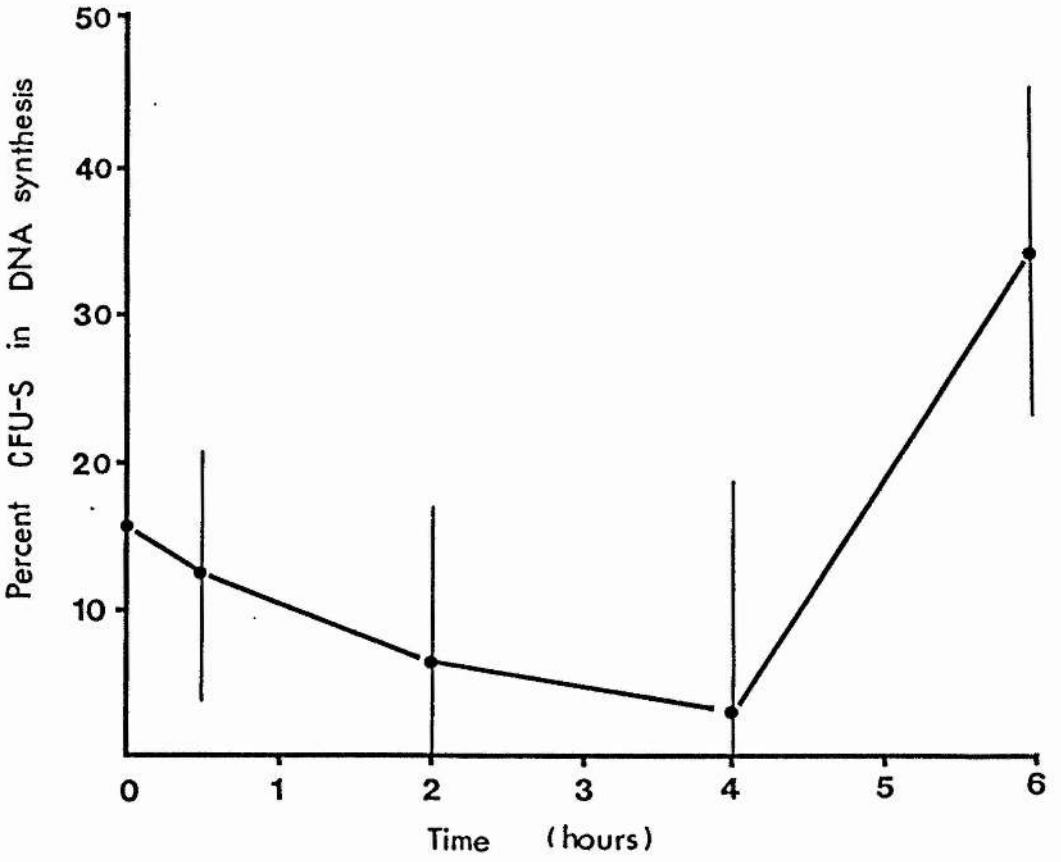

Figure 3.4.2: Percentage of femoral CFU-S in DNA synthesis (mean±SE of single experiments) following 4.50 Gy of whole body X-irradiation.

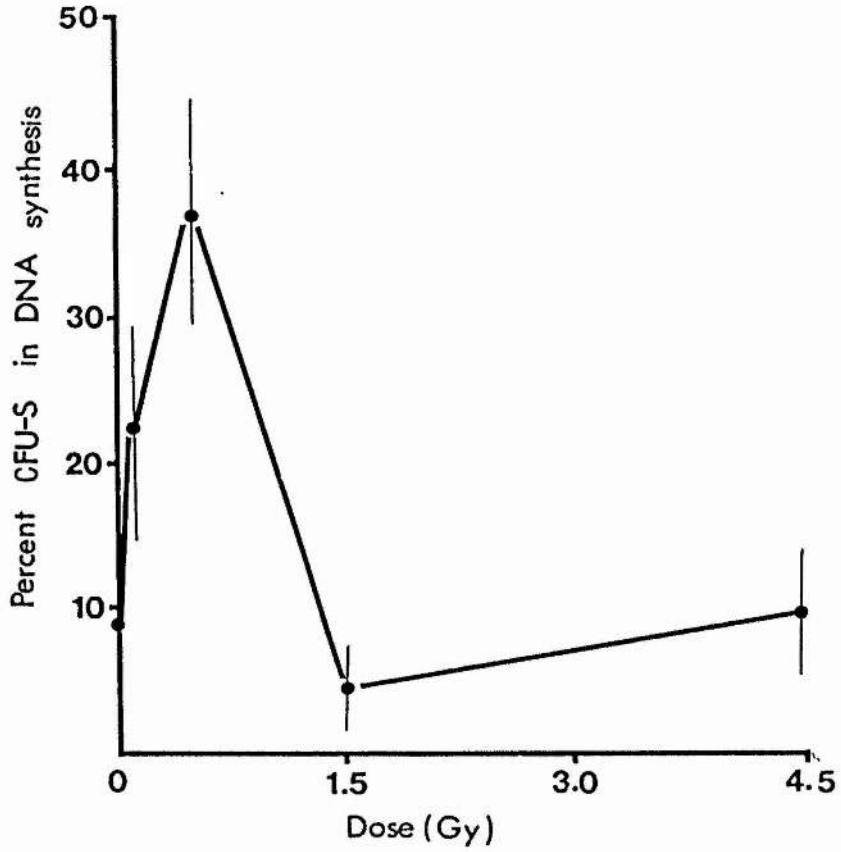

Figure 3.4.3: Percentage of femoral CFU-S in DNA synthesis (mean±SE of single experiments) 2 hours following 0.10 Gy, 0.50 Gy, 1.50 Gy and 4.50 Gy.

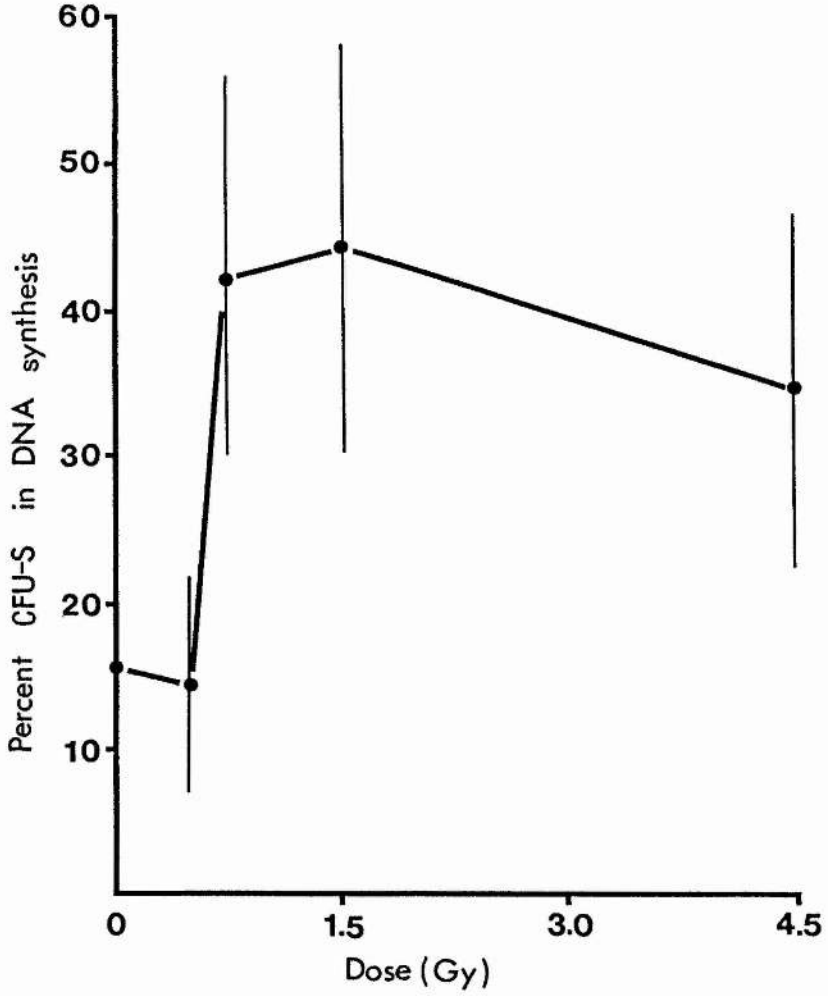

Figure 3.4.4: Percentage of femoral CFU-S in DNA synthesis (mean±SE of single experiments) 6 hours following 0.50 Gy, 0.75 Gy, 1.50 Gy and 4.50 Gy.

Results

The percentage of CFU-S in DNA synthesis was increased to 37.0±7.0 percent at 30 minutes and 43.9±11.2 percent at two hours after 0.50 Gy whole body X-irradiation (figure 3.4.1). However, the percentage decreased to 23.3±9.9 percent at 4 hours and returned to control levels after 6 hours (14.8±8.1 percent). The number of CFU-S per 10^5 cells injected was decreased to 65-76 percent of control (table 3.4a).

At sub-lethal dose of 4.50 Gy, the CFU-S population did not enter into DNA synthesis until 6 hours. The percentage of CFU-S in DNA synthesis was the same as control levels between 30 minutes to 4 hours after exposure. After 6 hours the percentage of CFU-S in DNA synthesis increased to 34.0±14.0 percent (table 3.4b). The proportion of CFU-S in DNA synthesis measured two hours after exposure to whole body X-irradiation was increased and reached 30-50 percent at a dose of upto 0.50 Gy. Above this dose the proportion was at control levels (figure 3.4.3). At 6 hours after 0.75 Gy, 1.5 Gy and 4.5 Gy doses the percentage of CFU-S in DNA synthesis was increased to 30-40 percent (figure 3.4.4).

The CBA/H mice exhibited the same response as CD1 mice after 0.50 Gy and 1.50 Gy whole body X-irradiation (table 3.4c&d). Both strains showed fast triggering into DNA synthesis at 30 minutes after exposure to the 0.50 Gy. The percentage of CFU-S in DNA synthesis was 53.3±26.7 for CBA/H mice and 37.0±7.1 for CD1 mice (table 3.4c).

3.5 Changes in the proportion of CFU-S in DNA synthesis after in-vitro irradiation.

Experimental procedure.

Bone marrow plugs were placed on a millipore filter and irradiated on a stainless steel grid in a petri dish containing medium at 0.50 Gy or 4.50 Gy doses (diagram 1). After irradiation the plugs were incubated in an incubator at 37°C with an air plus 10 percent CO_2 atmosphere. After 2 hours or 6 hours incubation the bone marrow plugs were broken up into single cell suspensions and the percentage of CFU-S in DNA synthesis was measured.

Results.

The number of CFU-S in the plug of bone marrow was decreased when incubated for the longer periods. After two hours incubation, the number of colonies per 10^5 cells injected was 25.0 ± 2.0 and after 6 hours incubation the number was decreased to 16.8 ± 2.8. The proportion of CFU-S in DNA synthesis had not increased after two hours and six hours incubation in-vitro. Following irradiation at 0.50 Gy dose to the plug of bone marrow

and 2 hour incubation, the proportion of CFU-S in DNA synthesis was increased to 27±13.3 percent. A similar increase was observed when the plug of bone marrow was exposed to 4.5 Gy and incubated for 6 hours (table 3.5).

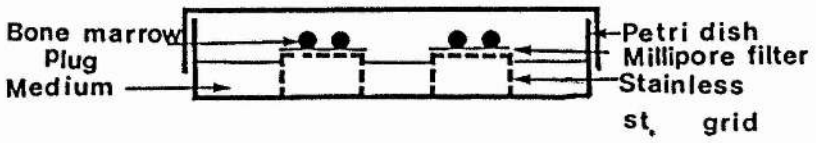

Diagram 1: Irradiation of bone marrow plugs in-vitro.

Table 3.5: Percentage of CFU-S in DNA synthesis (mean±SE of single experiments) after irradiation in-vitro.

Dose of irradiation	Time after incubation (hours)	CFU-S per 10^6 cells injected + medium	+ ARA-C	% CFU-S in DNA synthesis
Control	2	250±20	210±35	3.5±16.1
0.50 Gy	2	157±20	115±15	27±13.3
Control	6	167.5±27.5	155±27.5	7.5±16
4.50 Gy	6	15.3±1.9	10.2±1.3	33.8±10.0

3.6 The effect of supernatant and fraction III (30 - 50K) CM bone marrow of irradiated mice 7 days after 4.50 Gy.

Experimental procedure.

CM prepared from the bone marrow of irradiated mice 7 days after 4.50 Gy and the 30 - 50K Amicon fraction III of CM were prepared as described in the materials and methods section. The presence of stimulator of CFU-S proliferation in the CM and fraction III were tested by incubating with normal bone marrow for 2 hours at 37°C and measuring the percentage of CFU-S in DNA synthesis.

Results.

Incubation of normal bone marrow with CM prepared from bone marrow from irradiated mice 7 days post 4.50 Gy increased the percentage of CFU-S in DNA synthesis from less than 10 percent to 30 percent. A similar increase was also observed when Amicon fraction III (30 - 60 $\mu g\ ml^{-1}$) was incubated with normal bone marrow (figure 3.6.1). The number of CFU-S per 10^5 cells injected was slightly increased after incubation with Amicon fraction III but not significantly ($P > 0.1$).

Table 3.6: The percentage of CFU-S from normal bone marrow in DNA synthesis (mean±SE of single experiments) after incubating with 30 $\mu g\ ml^{-1}$ and 60 $\mu g\ ml^{-1}$ of fraction III 4.50 Gy bone marrow.

Concentration of fraction ($\mu g\ ml^{-1}$)	Colonies per 10^5 cells injected	% CFU-S in DNA synthesis
Normal bone marrow	18.6±2.0	3.3±15.6
30	22.0±2.8	38.2±9.0
60	21.0±1.8	34.3±8.7

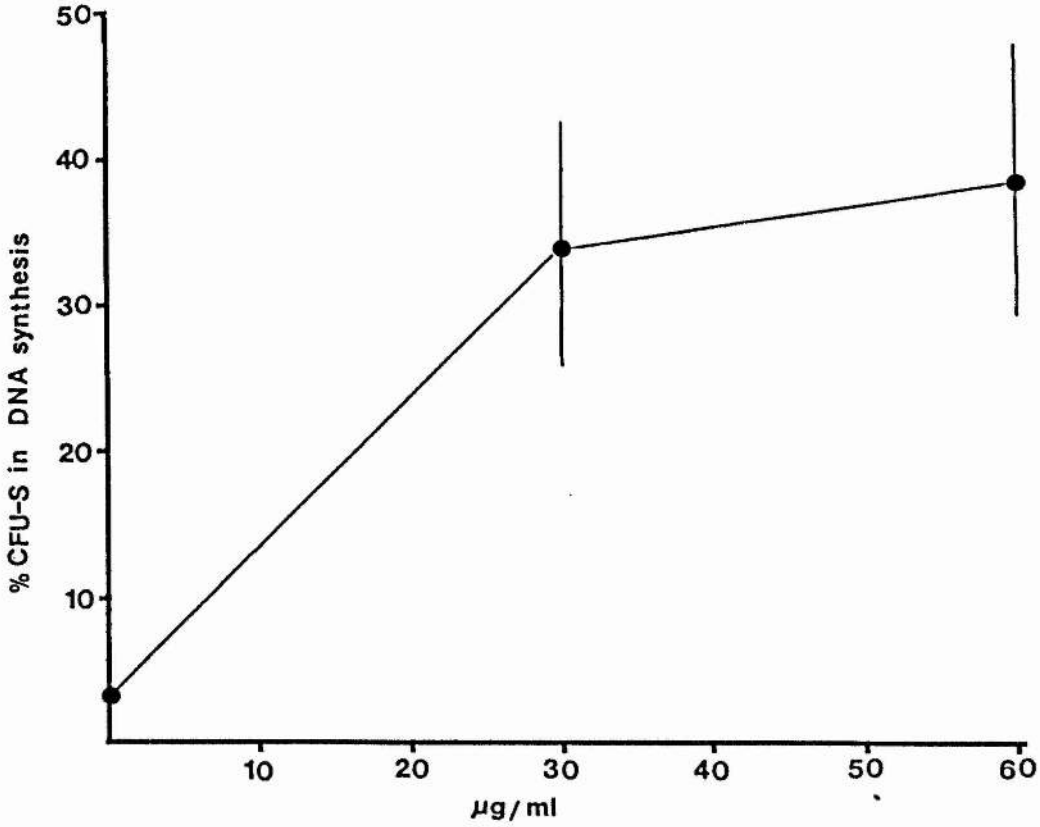

Figure 3.6.1: The effect of the 30 - 50k Amicon fraction III from 4.50 Gy day 7 bone marrow on CFU-S proliferation.

3.7 The effect of stimulator on CFU-S of mice irradiated at 1.50 Gy one hour after exposure.

Experimental procedure.

Female CD1 mice were irradiated with a 1.50 Gy dose and a bone marrow cell suspension was prepared 30 minutes after exposure. Cells were washed once with Fischer's medium and spun down at 1000 rpm for 10 minutes and resuspended with CM of bone marrow or 30 - 50K Amicon fraction III and incubated for 2 hours at 37°C. The percentage of CFU-S in DNA synthesis was than measured by ARA-C suicide technique(see materials and methods section 2.9).

Results.

The percentage of CFU-S in DNA synthesis from bone marrow 30 minutes after 1.50 Gy was less than 10 percent (table 3.7). Incubation with CM from bone marrow 4.50 Gy (7 days post irradiation) or medium for two hours however did not increase the proportion of CFU-S in DNA synthesis. Table 3.7 shows that the percentage of CFU-S in DNA synthesis from normal bone marrow was 32.3 ± 16.0 and CFU-S from 1.50 Gy bone marrow was 6.25 ± 33.2.

Table 3.7: The percentage of CFU-S in DNA synthesis (mean±SE of single experiments) from normal bone marrow and bone marrow irradiated mice at 1.50 Gy after incubating with CM from bone marrow irradiated mice at 4.50 Gy.

Experiment	CFU-S per 10^6 cells injected	% CFU-S in DNA synthesis
1		
Nbm	244±28	13.1±16.6
Nbm + CM 4.50 Gy bm	220±32	32.3±16.0
1.50 Gy + CM 4.50 Gy bm	12.8±4.0	6.25±33.2
2		
Nbm	242±14	7.4±9.2
1.50 Gy + medium	17.0±1.5	14.1±10
1.50 Gy + CM 4.50 Gy bm	12.1±1.8	5.8±17.5

3.8 The presence of a stimulator of CFU-S proliferation in bone marrow of mice irradiated at low or sub-lethal doses of whole body X-irradiation.

Experimental procedure

Female CD1 mice were irradiated at 0.50 Gy or 4.50 Gy of whole body X-irradiation and the conditioned media from bone marrow were prepared at the following time: 30 minutes, 2 hours and 6 hours after exposure. The presence of a stimulator in CM were tested by incubating with normal bone marrow for 2 hours and the percentage of CFU-S in DNA synthesis was measured.

Results.

Incubation of normal bone marrow CM with normal bone marrow did not increase the proportion of CFU-S in DNA synthesis (table 3.8a). Following 0.50 Gy whole body X-irradiation the stimulator of CFU-S proliferation was present in CM prepared 2 hours after exposure but not at 6 hours after. Table 3.8a shows that the percentage of CFU-S in DNA synthesis of normal bone marrow was increased to 53.4±7.9 percent after incubating with CM prepared

at 2 hours after 0.50 Gy dose.

Following the 4.50 Gy whole body X-irradiation dose, the stimulator was found in CM from bone marrow at 6 hours after exposure. The percentage of CFU-S in DNA synthesis from normal bone marrow was increased from 9.0±2.0 to 32.6±1.0 percent after incubation with 50 percent concentrations of CM from bone marrow 4.50 Gy dose prepared at 6 hours. However incubation with 75 to 80 percent CM prepared 30 minutes to 4 hours after exposure did not increase the proportion of CFU-S from normal bone marrow in DNA synthesis (see table 3.8b).

Table 3.8a: The effect of CM prepared from bone marrow from irradiated mice at 0.50 Gy whole body X-irradiation on CFU-S proliferation.

Time after irradiation (hours)	Concentration of CM (%)	CFU-S per 10^5 cells injected	% CFU-S in DNA synthesis
Nbm	-	29.6±2.2	8.1±10.0
2	75	29.5±3.4	53.4±7.9
6	75	29.5±2.9	9.6±2.0

Table 3.8b: The effect of CM prepared from bone marrow from irradiated mice at 4.50 Gy whole body X-irradiation on CFU-S proliferation.

Time after irradiation (hours)	Concentration of CM (%)	CFU-S per 10^5 cells injected	% CFU-S in DNA synthesis
Nbm	-	25.0±1.0	6.4±2.9
0.5	75	24.6±2.6	9.0±12.0
2	80	24.6±2.6	7.0±14.0
4	86	24.2±2.0	8.2±14.0
6	50	27.0±2.5	32.0±1.0

3.9 The effect of TCGF (interleukin II) and PEC-CM on the CFU-S proliferation.

Experimental procedure.

TCGF was provided by Dr.Jacob (Cambridge) and PEC-CM were prepared by incubating the peritoneal exudate cells in Fischer's medium for 3 hours (see materials and methods section 2.3.3). TCGF in volumes of 1.5 ml was incubated with one ml of normal bone marrow cells at a concentration of 5×10^6 cells ml^{-1} for 2 hours at 37^oC. An aliquot of 0.5 ml of PEC-CM (prepared at a cell concentration of 9×10^6 cell ml^{-1}) was incubated with 0.50 ml of 3×10^6 cells ml^{-1} for 2 hours at 37^oC. The percentage of CFU-S in DNA synthesis was measured at the end period of incubation (see materials and methods section 2.9).

Table 3.9a: The effect of TCGF on CFU-S proliferation.

Experiment	Colonies per 10^5 cells injected	% CFU-S in DNA synthesis
1		
Nbm	27.1±2.7	0.0±11.4
Nbm + TCGF	32.4±2.3	24.6±11.8
2		
Nbm	20.6±1.8	19.4±11.2
Nbm + TCGF	20.6±1.8	11.7±11.7
3		
Nbm	31.4±2.4	13.4±11.1
Nbm + TCGF	34.8±2.2	17.4±8.6

Table 3.9b: The effect of PEC-CM on the CFU-S proliferation.

Experiment	Colonies per 10^5 cells injected	% CFU-S in DNA synthesis
1		
Nbm	31.6±2.4	13.3±11.0
Nbm + PEC-CM	24.1±2.2	13.4±10.9
2		
Nbm	34.6±2.8	10.0±11.2
Nbm + PEC-CM	23.4±3.8	12.4±24.6

Results.

Experiment 1 (table 3.9a) shows that a significant increase of CFU-S in DNA synthesis from normal bone marrow after 2 hours incubated with TCGF. The percentage of CFU-S in DNA synthesis was increased from 0.0 ± 11.4 to 24.6 ± 11.8 percent. However in repeat experiments no effect on CFU-S proliferation was observed. In experiment 1 (table 3.9a), the number of CFU-S per 10^5 cells injected did increase ($P > 0.1$) but not in experiment 2 (table 3.9a).

Incubation of PEC-CM with normal bone marrow at a concentration of 75 percent has no effect on the CFU-S proliferation. However the number of CFU-S per 10^5 cells injected decreased to 68 - 76 percent as compared to normal bone marrow incubated with medium ($0.05 > P > 0.01$).

3.10 Characteristic of stimulator producing cells.

Experimental procedure.

3.10.1 Radioresistant cell population.

Mice were irradiated at 9.0 Gy whole body X-irradiation and CM from bone marrow were prepared at one, two, three and five days after exposure. The presence of stimulator in CM was then tested by incubating with normal bone marrow and measuring the percentage of CFU-S in DNA synthesis.

3.10.2 Thy 1.2, Fc and H-2k characterization.

CBA/H mice were irradiated at 4.50 Gy whole body X-irradiation and 7 days after the Thy 1.2^+ cells and Ia-$2k^+$ cells in bone marrow were eliminated (see materials and methods section 2.13.1 and 2.13.2). The Fc^+ cells were obtained by separating the rosettes from single cells by using discontinuous

BS gradients (see materials and methods section 2.12.5). After treatment the bone marrow cells were then incubated for 3 hours and prepared for CM. The presence of stimulator was than tested by incubating with normal bone marrow. The proportion of $Thy1.2^+$ cells, Fc^+ cells and $Ia\text{-}2k^+$ cells were also estimated.

Table 3.10a: The effect of CM from bone marrow of mice irradiated at 9.0 Gy whole body X-irradiation on CFU-S proliferation (mean±SE of single experiments)

Time after irradiation (day)	Concentration of CM (%)	CFU-S per 10^5 cells injected	% CFU-S in DNA synthesis
Nbm	-	24.4±2.0	7.4±12.0
1	67	24.0±2.0	37.5±7.8
3	67	28.0±2.8	33.6±9.8
5	67	20.4±1.2	49.6±9.9

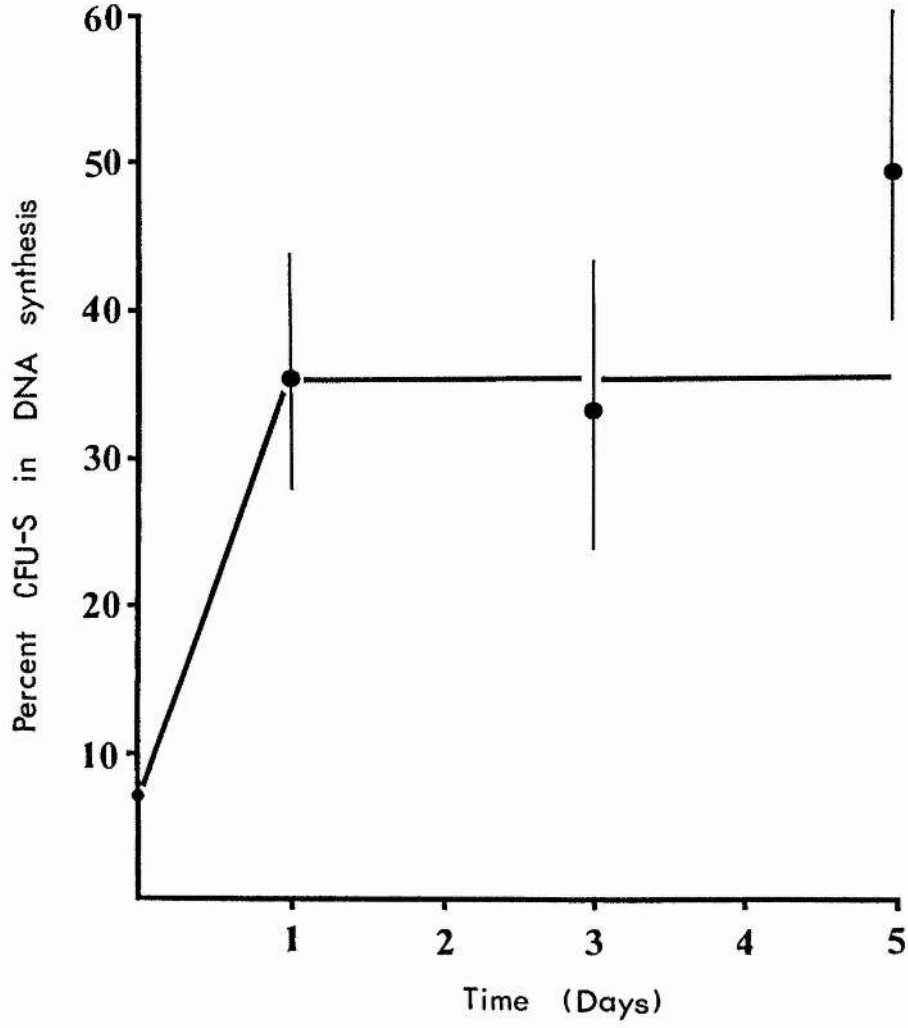

Figure 3.10: The effect of CM from bone marrow of mice 1 to 5 days post 9.0 Gy whole body X-irradiation on CFU-S proliferation.

Table 3.10b: The effect of CM from 4.50 Gy bone marrow (Thy 1.2$^-$) on CFU-S proliferation (mean±SE of single experiments).

Experiment	Colonies per 10^5 cells injected	% CFU-S in DNA synthesis
1		
Nbm	26.0±1.8	11.5±8.7
Nbm + Thy 1.2$^-$ CM	21.0±1.8	31.1±5.1
2		
Nbm	26.8±2.3	6.7±10.4
Nbm + Thy 1.2$^-$ CM	20.8±1.9	26.0±13.0

Table 3.10c: The effect of Fc^+ and Fc^- of CM of 4.50 Gy bone marrow on CFU-S proliferation (mean±SE of single experiments).

Experiment	Colonies per 10^5 cells injected	% CFU-S in DNA synthesis
1		
Nbm	24.9±2.4	5.9±11.3
Nbm + Fc^- CM	23.3±2.6	4.5±3.2
2		
Nbm	32.0±2.3	8.0±9.6
Nbm + Fc^- CM	33.0±3.2	2.2±12.6
Nbm + Fc^+ CM	32.4±3.2	26.4±9.0
3		
Nbm	35.5±2.4	13.0±7.6
Nbm + Fc^- CM	32.0±2.1	10.4±8.8
Nbm + Fc^+ CM	30.0±0.9	35.0±6.4

Table 3.10d: The effect of CBA/H-CM and C57BL10-CM of 4.50 Gy marrow on CFU-S proliferation (mean+SE of single experiments).

Experiment	Colonies per 10^5 cells injected	% CFU-S in DNA synthesis
1		
Nbm	21.6±2.4	16.6±13.1
Nbm + CBA/H-CM	23.6±2.6	14.4±13.3
Nbm + C57BL10-CM	29.4±2.2	29.3±7.5
2		
Nbm	20.0±2.2	18.0±12.7
Nbm + CBA/H-CM	23.6±1.4	9.3±11.5
Nbm + C57BL10-CM	18.4±2.4	38.0±11.1
3		
Nbm	21.8±2.6	12.4±12.3
Nbm + CBA/H-CM	20.2±2.2	0.0±14.6
4		
Nbm	21.6±2.4	17.0±11.9
Nbm + C57BL10-CM	20.2±2.4	30.3±15.3

CBA/H-CM - conditioned media of bone marrow of CBA/H (Ia-$2k^+$) prepared after cytolysis of Ia-$2k^+$ cells.

C57BL10-CM - conditioned media of bone marrow of C57BL10 (Ia-2b) prepared after incubation with anti Ia-2k and compliment.

Results.

The number of nucleated cells in femoral marrow was reduced to 15±9 percent of control one day after 9.0 Gy whole body X-irradiation. It was further decreased to 1.5±8 and 1.1±8 percent of control 3 and 5 days after. The stimulator of CFU-S proliferation was present in bone marrow one to five days after irradiation. The proportion of CFU-S in DNA synthesis of normal bone marrow increased to about 33.6±9.8 - 49.6±9.9 percent after 2 hours incubation with CM from bone marrow from irradiated mice at 9.0 Gy one to five days post irradiation (figure 3.10).

Regenerating bone marrow 7 days post 4.50 Gy whole body X-irradiation contains of 2.3±1.0 percent of Thy 1.2^+, 34.0±2.0 percent of Fc^+ and 3±0.6 percent of $Ia\text{-}2k^+$ cells. Elimination of Thy 1.2^+ population by complement mediated cytolysis did not affect the capacity of regenerating bone marrow to produce the CFU-S proliferation stimulator. The number of CFU-S per 10^5 cells injected was decreased after incubation with CM of bone marrow cells of Thy 1.2^- (table 3.10b).

Elimination of Ia-2k$^+$ cell population however did affect the capacity of regenerating bone marrow to produce stimulator. Table 3.10d shows that the CM from regenerating bone marrow of CBA/H prepared after complement mediated cytolysis of Ia-2k$^+$ cells did not change the proportion of CFU-S normal bone marrow in DNA synthesis. However the CM of C57BL/10ScSn/01a (Ia-2b) did increase the percentage of CFU-S in DNA synthesis to about 29.3$\pm$7.5 - 38.0$\pm$11.1 percent (table 3.10d).

Cells that formed rosettes with sensitized erythrocytes were similarly active in producing the stimulator which increased the proportion of CFU-S from normal bone marrow in DNA synthesis from less than 10 percent to 30 percent. Media prepared from the non-rosette forming fraction were not stimulatory to CFU-S proliferation; 2 hours incubation with these media at 67 percent concentration did not increase the proportion of CFU-S in DNA synthesis which remained at 10 percent (table 3.10c).

DISCUSSION

CONTENTS

CHAPTER 4

DISCUSSION

4.1 The recovery of CFU-S population following whole body X-irradiation

The CFU-S population in bone marrow or spleen was capable of recovering to normal levels by about 4 weeks after sub-lethal dose of whole body X-irradiation (Guzman and Lajtha, 1970; Hendry and Lajtha, 1972). However there are also reports showing that the CFU-S numbers unable to recover to normal levels after a single sub-lethal dose (Carsten and Bond 1979; Covelli and Metalli 1973; Croizat et al. 1979). Furthermore in mice exposed to 0.70 Gy/day for a period of 49 days (Wu and Lajtha, 1975) and repeated irradiation (Hendry and Lajtha, 1972) the CFU-S population in femur and spleen recovered only to sub-optimal levels.

Recently Schofield and Dexter (1982) reported that the CFU-S number in femoral marrow of BDF1 mice did not rapidly recover to normal level after exposure to a dose of 0.5 Gy. The number of CFU-S per femur was shown to decrease to about 60 percent of control values. The post irradiation dip was also observed between 1 to 3 days after irradiation, whereby the CFU-S number was further decreased to about 30 to 40 percent of normal levels. The mechanism of the dip is unknown. However it has been postulated that it may be result from a direct effect of radiation on CFU-S differentiation (Lahiri et al. 1970) or from a normal removal of CFU-S by differentiation at normal rate during period of mitotic delay after irradiation (Wu et al. 1975). A similar dip also been observed when unirradiated CFU-S were transplanted into lethally irradiated recipients. Hendry and Lord (1983) suggested that this was due to the heterogeneity in self-renewal capacity of CFU-S in which CFU-S with low self-renewal capacity will be lost due to the demand of differentiation and only fraction of CFU-S with high self-renewal will recover.

In this work with the same dose level as Schofield and Dexter (1982), the CD1 mouse strain did not exhibit delayed recovery. The number of CFU-S in the femur did not change markedly following 0.5 Gy dose but at a sub-lethal dose of 4.5 Gy the effect was the same as reported by Hendry and Lajtha, (1972) and also no differences were observed between CD1 and CBA/H mouse strains. However the depletion of GM-CFC in femur following 0.5 Gy in this work was similar to that observed by Schofield and Dexter (1982).

The seeding efficiency of CFU-S post 0.5 Gy however was not measured in Schofield and Dexter's (1982) work. The correction for the change of seeding efficiency is very important in measuring the recovery of CFU-S in absolute numbers after irradiation. The seeding efficiency was reported reduced when mice were treated with various cytotoxic agents such as endotoxin, vinblastine and sub-lethal irradiation (Fred and Smith, 1968). Furthermore Necas (1981) showed that the number of CFU-S per femur was decreased after a single dose of hydroxyurea. However when the numbers were corrected for the change of seeding efficiency it was shown that an over shoot occurs after 18

to 20 hours. At the other time interval the seeding efficiency of CFU-S was not changed significantly. However treatment with a single dose of colchicine the seeding efficiency was not changed significantly (Necas, 1981).

Furthermore Monette and De Mello (1979) have shown that the seeding efficiency of cycling CFU-S was decreased to about 50 percent as compared to non-cycling CFU-S. However this observation was not consistent with the experiments reported by Dumenil et al. (1984). They have shown that no difference between the seeding efficiency of queiscent CFU-S from normal bone marrow and cycling CFU-S after ARA-C treatment. Furthermore Gidali et al. (1974) reported that peripheral CFU-S were described to be in cycle but their seeding efficiency was higher than CFU-S of normal bone marrow. Seeding efficiency of CFU-S induced into cycle in-vitro with interleukin-3 also was reported unchanged as compared to non-cycling CFU-S (Garland and Crompton, 1983).

The prolongation of CFU-S recovery following 0.5 Gy dose in Schofield and Dexter's (1982) experiments might be due to the effect of radiation on the CFU-S population and also on the microenvironment. These effects were shown by

Hendry and Lajtha (1970) when repeated whole body X-irradiation was performed but not after a single dose. The rate of CFU-S recovery following repeated doses was found lower than after a single dose. When the CFU-S from repeatedly irradiated mice were transplanted into lethally irradiated recipients they only colonize the femur at the sub-optimal level. However the rate of growth was the same as the unirradiated CFU-S.

The microenvironment of the femoral marrow has been shown to be more radioresistance than the CFU-S which are contained in the marrow (Chamberlin et al. 1974; Freid et al. 1976). The influence of the CFU-S microenvironment on proliferation of CFU-S could be assayed by implanting a femur under into subcutenous tissue and after six weeks the numbers of CFU-S in the femur were estimated (Freid et al. 1976). In mice irradiated at 5.0 Gy or less, the microenvironment for CFU-S proliferation was found unchanged. At this dose more than 99 percent of CFU-S were killed (Chamberlin et al. 1974). Furthermore Chamberlin et al. (1974) demonstrated that after 10 Gy exposure the stroma in the femur still supported repopulation and recovery of CFU-S number to 1/3 of grafted control femur

levels ensued.

In studies using long term bone marrow cultures, the sensitivity of adherent layers to radiation however appears to be inconsistent with these findings. For example Dexter et al. (1978) reported that the ability of adherent layers to support CFU-S growth was diminished when exposed to 0.25 Gy and destroyed when irradiated at 0.5 Gy dose. The effects on the adherent layers however have not been detected at a morphological level and they presumed to reflect a sensitive component of cell interactions (Dexter et al. 1978). In other experiments reported by Mori et al. (1981) shown that the CFU-S in bone marrow culture were able to recover as in-vivo following exposure to the dose between 0.5 Gy to 3.0 Gy of X-irradiation.

Moreover Dexter et al. (1978) also reported that the adherent layers produced from bone marrow of mice irradiated at 6-7 Gy grow poorly and to be deficient in fat cells, endothelial cells and not supporting the growth of CFU-S. However this contrasts to the observation of Song and Quensenberry (1984); they have shown that the adherent cells from mice irradiated 24 hours previously at 10 Gy dose

capable of supporting CFU-S and GM-CFC cells derived from the non-adherent cells of other long term marrow cultures. Two types of cells were shown to establish in the adherent layers when bone marrow cells of mice irradiated at 10 Gy were cultured. They are a macrophage like cell and epitheloid like cell.

Furthermore Dexter et al. 1979 also reported that bone marrow of repeatedly irradiated mice also failed to establish adherent layers and the CFU-S population obtained from repeatedly irradiated mice did not survive long on a normal unirradiated adherent layer (Dexter et al. 1979).

4.2 CFU-S heterogeneity and respon. to regulator factors.

The CFU-S population has been shown to be a heterogenous population in respect to self-renewal capacity (Worton et al. 1969; Schofield and Lajtha, 1974; Schofield et al. 1980) (see section 1.1.8). Furthermore it was suggested by Schofield (1978) and Rosendaal et al. (1979) that the CFU-S population is organized on the basis of an age structure in which young CFU-S with high self-renewal capacity during subsequent divisions produced older CFU-S with decreasing self-renewal capacity and increasing differentiation potential.

The spleen colony assay is an operational assay to detect cells which can form colonies on the spleen of lethally irradiated mice. During the transplantation of bone marrow cells into a lethally irradiated recipient, the CFU-S with different self-renewal capacity are randomly seeded into the spleen. Some of these cells could not not reach the spleen but settled in other haemopoietic tissue such as bone marrow and some might be lost due to seeding in an unsuitable environment or due to terminal

differentiation. Some of the colonies established by low self-renewal potential CFU-S will thus run out of stem cells.

The spleen colony assay can be performed between 7 to 14 days. Most of the spleen colonies observed at 7 to 9 days consisted predominantly of recognizable erythroblasts (Curry and Trentin, 1967) and the colonies observed at 14 days contained cells of more than one cell line (Siminovitch et al. 1964). It has been suggested that the character of spleen colonies from unilineal at early times was transformed into mixed colonies later due to the local operation of instructive differentiative signal in the splenic environment (Curry and Trentin, 1967).

Recently this suggestion has been questioned and shown not to be true for all the colonies. Magli et al. (1982) showed that not all the colonies observed at 7 or 8 days remained at 10 to 12 days. On the basis of longitudinal studies between 7 to 12 days they have shown that there were three types of colonies: transient, persistent and delayed in appearance. Most of the 7or 8 days colonies consisted predominantly of recognizable erythroblasts and lacked

committed progenitor cells whereas most of 11 or 12 days colonies are multilineal and contained committed progenitor cells. Half of the colonies visible on day 7 or 8 would be visible on day 10 or 12 and one-third of the colonies visible on day 10 had visible antecedents on day 7 or 8. They concluded that the spleen colony assay measures pluripotential cells capable of extensive proliferation only when colonies are scored at the later time points. Wright et al. (1985) however suggested that Magli et al. (1982) observations are compatible with an age structure of CFU-S population in which older CFU-S are assayed as day 7 CFU-S and younger CFU-S with high self-renewal capacity are detected when spleen colonies are scored at later times.

The proliferative rate of CFU-S from normal or regenerating marrow measured by ARA-C or ^{3}HTDR suicide technique however show no differences between velues assayed at day 7 or day 12. The percentage of CFU-S in DNA synthesis of normal bone marrow was about 10 percent and for regenerating CFU-S the percentage are about 30 to 40 percent (Wright et al. 1985). However Chertkov and Drize (1984) showed that the suicide value of CFU-S from sub-lethally irradiated mice assayed at 7 days were different from the

one assayed at 11 days. A high proportion of CFU-S were shown to be killed by S-phase cytotoxic agents when assayed at 7 days while only a low kill was observed at day 11. This value was however obtained from an in-vivo suicide method only and the suicide values for CFU-S from normal bone marrow assayed at 7 days and 11 days were not measured.

Chertkov and Drize (1984) also showed that the value of in-vivo and in-vitro suicide of CFU-S from lethally irradiated mice reconstituted with syngeneic bone marrow cells scored at 7 or 11 days are the same. In Chertkov and Drize (1984) study the number of CFU-S observed per spleen for both the in-vivo and in-vitro suicide of CFU-S from lethally irradiated mice reconstituted with syngeneic bone marrow cells scored at later times was half of the number of CFU-S observed at 7 days. On the other hand Wright et al. (1985) reported that there was no significant different between the number of colonies observed at 7 days or 12 days both for CFU-S from normal bone marrow or regenerating bone marrow following X-irradiation.

The response of CFU-S assayed at 7 days and 12 days post transplantation to the different concentration of inhibitor and stimulator of CFU-S proliferation was studied by Wright et al. (1985). They have shown that day 12 CFU-S of normal bone marrow were less sensitive to stimulator than day 7 or day 10 CFU-S. On the other hand the inhibitor was shown more effective against day 12 CFU-S than day 7 CFU-S. These observation are compatible with an age-structure in the CFU-S compartment in which there is a continuum of cells with decreasing capacities for self-renewal and increasing for differentiation (Hellman et al. 1978).

4.3 Regulation of CFU-S proliferation following whole body X-irradiation

The proportion of CFU-S in the medullary cavity of normal CD1, CBA/H and C57BL/10ScSn/Ola mice killed by ARA-C treatment was about 0 to 15 percent. This value was similar to those reported earlier by Becker et al. (1965) and Lord et al. (1974) using ^{3}HTDR. A high kill of CFU-S from normal bone marrow was reported by Necas et al.(1981). This might be due to the physiological changes of the mouse which increase the proportion of CFU-S in DNA synthesis.

In normal steady state the CFU-S population in normal bone marrow was suggested to be out of the cell cycle and in a resting state called the Go state (Lajtha, 1979).

The inhibitor and stimulator of CFU-S proliferation were shown to act as an on/off switch of proliferation (Lord, 1979). The stimulator was capable of inducing quiescent CFU-S into DNA synthesis within about a half-an-hour incubation. For the inhibitor it was shown that a minimun threshold dose for effective inhibition varies inversely with the duration of exposure to the

inhibitor. Lord et al. (1979) suggested that there are two posibilities that the inhibitor exert its effect on CFU-S at the threshold dose. The first is that the inhibitor is effective only at the early part of the G1 phase and this permits the G1 cells to progress normally into S-phase and therefore prevents an immediate observation of inhibition. As the dose of inhibitor is increased the effect of inhibitor is extended throughout the G1-phase and to the G1-S boundary, this will effectively block the entry of cells into the S-phase and thus result in immediate inhibition. The second suggestion is that the threshold dose of inhibitor is effective in the early G2 period which allows the affected cells to continue their progress to the end of the cell cycle. The inhibition therefore is not seen until this time has elapsed. Higher doses of inhibitor affect later G2 cells which results in an early recognition of the block.

The location of the position of Go for CFU-S in the cell cycle was suggested to occur at three possible locations: at the boundary of G1, early G1 or the boundary of G2-M by Lord (1981). By using different incubation combinations of inhibitor to induce Go block, vinblastine to

produce mitotic block and stimulator to restimulate the CFU-S into cell cycle Lord (1981) has shown that the Go position is either between the G1 and S-phase of the cell cycle or at the end or latter part of the G1-phase.

It was suggested that in the in-vivo situation the state of CFU-S proliferation was determined by the balance between the levels of inhibitor and stimulator present in the local environment (Lord et al. 1977; Wright et al. 1977). In in-vitro experiments it was shown that the effect of inhibitor and stimulator can override each other when appropriate concentrations are used (Lord et al. 1977). Also in experiments using long term marrow cultures it was shown that the addition of the inhibitor to the cultures can inhibit a highly proliferative CFU-S population present shortly after re-feeding. On the other hand the addition of the stimulator can increase the proliferation rate of quiescent CFU-S present seven days after feeding (Toksoz et al. 1980). Furthermore it has been shown that a high level of stimulator was present close to the bone surface where the CFU-S population was actively in DNA synthesis and in the centre of the bone marrow (axial region) high levels of inhibitor was present in normal control mice (Wright E. G.

personal communication 1981).

The quiescent CFU-S population can be triggered into DNA synthesis within 10 minutes after sub-lethal doses of 1.5 Gy of whole body X-irradiation (Lahiri and Van putten, 1970). In this work, the fast triggering of CFU-S into DNA synthesis only occurred when exposed to a low dose of 0.5 Gy. When the dose was increased the CFU-S population was delayed from entering into DNA synthesis. From the time course study, the CFU-S population was not in DNA synthesis until six hours after 0.75 Gy, 1.5 Gy and 4.5 Gy doses. This phenomenon was observed in both the CD1 and CBA/H mouse strains. In this work we have shown that the proportion of CFU-S in DNA synthesis in bone marrow measured two hours after exposure to whole body X-irradiation was increased and reached 30-50 percent at a dose of upto 0.5 Gy. Above this dose the CFU-S population was not stimulated at that time. However when the proportion of CFU-S in DNA synthesis was measured six hours after exposure, the values were the same as controls for doses less than 0.50 Gy and above this dose the values were 30 t0 50 percent.

In this study we also have shown that the presence of a stimulator of CFU-S proliferation in the bone marrow following whole body X-irradiation correlated with an increase of the proliferative status of CFU-S in the bone marrow. Lord and Wright (1982) have reported that the changes of the proliferative status of CFU-S in bone marrow of mice treated with PHZ between one to fifteen days was paralled with the presence or absence of stimulator activity.

The kinetic study of stimulator production in bone marrow following whole body X-irradiation also was carried out by workers in France (Croizat and Frindel, 1980). They have shown that the stimulator factor was present in bone marrow 15 minutes after 5.0 Gy whole body X-irradiation but not after two hours (Croizat and Frindel, 1980). In this work the stimulator was not detected in bone marrow of mice irradiated with 4.5 Gy dose until six hours.

The dose response curve of stimulator present in bone marrow 15 minutes after exposure using the in-vitro culture system showed that the stimulator level was increased with increasing dose and reached a maximun level at 5.0 Gy, above this dose the stimulator level was decreased as the dose increased to 9.0 Gy (Croizat and Frindel, 1980). However in this work when the stimulator was tested two hours after exposure, the activity was detected only in bone marrow of mice exposed to 0.5 Gy whereas when tested six hours after exposure the stimulator was present in bone marrow for the doses above 0.5 Gy. Furthermore the presence or the absence of stimulator was found parallel with the proliferative rate of CFU-S in bone marrow.

The differences between results which had been reported by Croizat and Frindel (1980) and results in this study might be attributed to the difference in techniques of assaying the stimulator. Croizat and Frindel (1980) used an in-vitro culture system whereby about six to eight plugs of bone marrows were floated on millipore filters and incubated with suspension of non-proliferating CFU-S as targets cells. After 24 hours incubation at 37^{o}C, the percentage of

CFU-S in DNA synthesis of target cells was then estimated by using thymidine suicide and the spleen colony assay. This technique was found not suitable for assaying stimulator or inhibitor because after 24 hours incubation only a few of the CFU-S target cells would have survived. Furthermore Croizat and Frindel (1980) did not use the regenerating bone marrow as a control and also they did not measure the percentage of CFU-S in DNA synthesis in bone marrow following 5.0 Gy whole body X-irradiation. However in the present study the conditioned media (supernatant free of cells) were used at an appropriate cell ratio with the responder cells (normal bone marrow). This method has been previously used by workers in Manchester and this laboratory, who have shown that stimulator factors in bone marrow or spleen can be eluted into the medium following short term incubation or washing of a cell suspension. When CM were incubated with normal bone marrow cells the percentage of CFU-S in DNA synthesis was increased to 30 - 50 percent (Lord et al. 1977; Wright et al. 1977; Cork et al. 1981; Cork et al. 1982; Riches et al. 1981a; Thomas et al. 1981).

By using the in-vitro plug assay system Croizat and Frindel (1980) have shown that in partial body irradiation at 5.0 Gy stimulator was shown present in irradiated bone marrow harvested 15 minutes after exposure but not in protected bone marrow. On the other hand when stimulator was tested two hours after exposure, the stimulator activity was detected only in protected bone marrow but not in irradiated bone marrow. Croizat and Frindel (1980) interpreted this observation as evidence for a long range control of CFU-S proliferation. However it was well established that the proliferation control of CFU-S occurred at local level. For example, Gidali and Lajtha (1974) reported that in partial body irradiation the number of CFU-S in protected areas decreased soon after exposure and the percentage of CFU-S in DNA synthesis markedly increase but returned to normal level after that. However in the irradiated marrow where the CFU-S population was markedly depopulated, the CFU-S population still continued to cycle rapidly. Furthermore Wright and Lord (1977) confirmed the observation of Rencricca et al. (1970) that seven days following phenylhydrazine, the CFU-S population proliferating maximally (30-50 percent) in the bone marrow

but at the same time in the spleen the rate was the same as control (see section 1.3.2). Moreover in bone marrow the CFU-S close to the bone surface were proliferatively active whereas in the axial region they were proliferatively quiscent(Lord, 1978).

The proliferation of CFU-S in-vivo was suggested to be determined by the balance of inhibitor and stimulator level (Lord et al. 1977). However the mechanism how this level was determined is not fully understood.

Lord and Wright (1980) proposed that the proliferative status of CFU-S might be regulated by the localized ratio of CFU-S number to inhibitor and stimulator producing cells. In normal steady state they have suggested that the presence of CFU-S produce a signal to the inhibitor producing cells by some unrecognized process. These cells respond by the production of inhibitor which keeps the CFU-S quiescent and also inhibits the stimulator producing cells from producing stimulator. If the CFU-S population is depleted the strong signal to the inhibitor producing cells is lost and thus the levels of inhibitor are reduced. This allows stimulator production to increase and blocks further

inhibitor production until the CFU-S population is restored and then the inhibitor production signal is back to normal.

However evidence from the literature illustrates that a high CFU-S is not necessarily associated with a low proliferative rate and, conversely a low CFU-S concentration is not always accompanied by a high proliferative rate. For example, following repeated partial body irradiation the numbers of CFU-S decreased slowly but continuously in the protected leg (Tubiana et al. 1977). In spite of this, the proportion of these cells in DNA synthesis returns to normal (less than 10 percent) at a time when there are less than 20 percent of the initial number of CFU-S in the whole body and about 10 percent in the in the protected leg. It also has been shown that for both normal bone marrow and regenerating marrow the highest proliferative rates of CFU-S are found in regions of highest CFU-S concentration (Lord, 1978). Therefore the proliferative status of CFU-S is not regulated simply by their total number, but it was suggested by the localised ratios of CFU-S number to inhibitor and stimulator producing cells (Lord and Wright, 1982).

From this study two phases of proliferative response of the CFU-S population were observed following whole body X-irradiation. The first was at low doses where the CFU-S were triggered into DNA synthesis within 30 minutes after exposure even though the CFU-S population was not significantly reduced. The second was at sub-lethal doses where the CFU-S were delayed in entering into DNA synthesis until six hours.

Fast triggering of CFU-S into DNA synthesis at 0.5 Gy could be explained by an effect on the cell surface membrane. Sato and Kojima (1972) reported that the negative surface charge of Burkitt lymphoma cells, mouse mammary carcinoma cells, mouse mammary carcinoma and mouse melanoma was decreased with time and reached a minimun at four hours after 0.5 Gy. This effect might result in changes in interaction between CFU-S with the microenvironment (inhibitor and stimulator producing cells) and produce a temporary signal to stop production of inhibitor and allow the production of stimulator. This signal however is temporary because there was no change in the CFU-S numbers which lead to increase in the production of inhibitor and

inhibit the production of stimulator. This effect results in the level of stimulator decreasing when measured at four hours and not being detected at six hours after exposure to 0.5 Gy dose.

However when the levels of doses were increased, the CFU-S population was shown to be delayed in entering into DNA synthesis. This might be due to damage to the receptor for the stimulator molecules on CFU-S after this dose of irradiation. In this study the CFU-S population obtained one hour after exposure to 1.5 Gy was shown not responsive to stimulator as CFU-S from normal bone marrow would be.

4.4 Characteristics of stimulator producing cells

The characteristics of stimulator producing cells were defined by the ability of cells in suspension to resynthese stimulator after removing or eliminating certain cell populations. Wright and Lord (1979) shown that regenerating bone marrow cell suspensions are capable of resynthesis of stimulator after reincubation for a period of at least two hours. On the other hand normal bone marrow cells are capable of resynthesis of inhibitor after reincubation for at least a period of five hours.

From cell fractionation studies by using bovine serum albumin density gradients, Wright and Lord (1978) showed that the CFU-S population is probably different from the inhibitor and stimulator producing cells in term of density. The distribution of CFU-S in normal bone marrow and regenerating bone marrow after PHZ was heterogeneous. Normal bone marrow shows maximum numbers of CFU-S around density of 1.070 gcm^{-3} and this peak is shifted to 1.076 gcm^{-3} in PHZ bone marrow. The inhibitor producing cell is concentrated in fractions with densities less than 1.060

gcm^{-3} which contain three percent of the total normal bone marrow CFU-S and about two percent of the PHZ bone marrow CFU-S. The stimulator producing cell on the other hand concentrated on the region of 1.061 - 1.076 gcm^{-3} which corresponds to about 20 percent of the total CFU-S in PHZ bone marrow and to about 40 percent in normal bone marrow. Thus only CFU-S sub-populations, if at all, would correlate with the producer populations.

The dose response curve for the effect of CM from PHZ bone marrow and from normal bone marrow can be compared in two ways. Results can be expressed on the basis of cell number or CFU-S number comparing the amount of CM used to the amount of target cell assayed. When cell number was used the curves are similar; if however CFU-S numbers are used there is a difference in the shape (Wright and Lord, 1978). Furthermore the concentration of stimulator was shown to be 10 times higher in the fractionated population as compared to the whole population of regenerating bone marrow. From these studies Wright and Lord (1978) suggested that the CFU-S population is not producing inhibitor or stimulator to regulate itself.

In this work the characteristic of stimulator producing cells was further defined as a continuation of Wright and Lord's work (1980). In this study the stimulator was shown to be produced by a radioresistant population. The aplastic bone marrow where the haemopoietic cells in bone marrow were very markedly depressed following 9.0 Gy whole body X-irradiation was shown to produce stimulator at day one to day five post irradiation. Wright et al. (1980) also reported that the inhibitor producing cell population was present in bone marrow of mice irradiated with 10 Gy whole body X-irradiation. This suggests that stimulator and inhibitor producer cells might be part of the haemopoietic environment which is not easily destroyed by radiation as compared to the haemopoietic cells themselves.

In long term bone marrow cultures, the maintenance of the CFU-S population is dependent upon the establishment of an adherent microenvironment population reprensentive of cells present in bone marrow stroma (Allen, 1981). The cellular source of the stimulator and also inhibitor of CFU-S proliferation resides in this adherent layer which contains endothelial cells, reticulum cells, fat cells, and

mononuclear phagocytes (Allen, 1981). The bone marrow of mice irradiated at 10 Gy whole body X-irradiation was also capable of forming adherent layers when cultured in-vitro and capable of supporting the CFU-S and GM-CFC populations (Song and Quensenberry, 1984).

The depletion of Thy 1.2^{+} cells from regenerating bone marrow has no effect on stimulator production. This result confirms the experiment reported by Wright et al. (1980). However in experiments using W/W^{V} mice, Wiktor-Jedrzejczak et al. (1977) and Sharkis et al. (1978) have shown that pretreatment of the marrow graft with Thy1.2 antisera prevents the restoration of normal erythropoiesis in these mice. Furthermore, the ratio of E/G colonies was changed from predominantly erythroid to granuloid but the colonies number were not changed. The addition of thymocytes to the Thy 1.2 treated bone marrow however will cure the anemia of W/W^{V} mice (Sharkis et al. 1978). This anti-theta-sensitive-regulatory cell (TSRC) has been shown to posseses Thy 1.2^{+}, Ly $1^{+}2^{-}3^{-}Ia^{-}$ markers (Wiktor-Jedrzejczak et al. 1979) and if not present will not allow survival of CFU-S in the W/W^{V} mouse. These regulatory cells are also present in 16-days fetal liver and

nu/nu bone marrow and would thus seem to develop without contact with mature thymic tissue (Harrison et al. 1979).

Thy 1.2^+ cells are also essential for the maintenance of the CFU-S population for longer periods. McMillan and Simmon (1981) showed that the addition of monoclonal antibody Thy 1.2^+ and rabbit complement to adherent layers of long term bone marrow cultures reduced the capacity of maintenance of the CFU-S population to four weeks. All these observations are consistent with the suggestion that Thy 1.2 cells are part of the microenvironment which is required to support CFU-S proliferation and differentiation but do not directly produce stimulator for CFU-S proliferation.

In this study we have shown that the stimulator producing cells are Fc^+, Ia-$2k^+$ and Thy 1.2^-. Lord and Wright (1980) also reported that the incubation of regenerating bone marrow cells with carbonyl iron followed by removal of the excess iron together with cells which ingested the iron would remove the cells which synthesize stimulator. The removal of adherent cells from regenerating bone marrow also removed the source of stimulatory activity

(Lord and Wright, 1980).

The accumulating data therefore implicate a marrow macrophage as the cellular source of the stimulator of CFU-S proliferation. However peritoneal macophages which are recruited from blood monocyte population (Van Furth and Cohn, 1968; Volkman and Gowans, 1965) failed to produce stimulator activities. Wright et al. (1980) also reported that the fraction IV (50 - 100K daltons molecular weight) of conditioned media prepared from several macrophage cell lines and peritoneal exudate cells did not produce an inhibitory effect on CFU-S proliferation. Furthermore Wright et al. (1980) showed that the inhibitor producing cells are Thy 1.2^{-} and Fc^{+} which are similar to the stimulator producing cells from this study. Wright et al. (1982) suggested that two different classes of macrophages are involved in producing the inhibitor and stimulator of CFU-S proliferation. Furthermore it has been suggested that they may be capable of producing both factor(s) (Wright et al. 1982).

However, Blackburn and Patt (1977) reported that the conditioned media obtained from bone marrow fibroblast cultures increased the number of CFU-S per 10^5 cells injected of normal bone marrow when incubated for 24 hours. This effect was decreased when the concentration of conditioned media was decreased. The same effect was also observed when the conditioned media from human bone marrow fibroblasts were used and also increased the proportions of CFU-S in DNA synthesis (Blackburn and Goldman, 1981). The relationship between these factor(s) derived from fibroblasts with the factor produced by macrophage like cells are not yet known. However it could also be speculated that the fibroblast derived factor(s) do not directly effect the CFU-S proliferation but stimulate the stimulator producing cells to produce stimulator and increase the proportion of CFU-S in DNA synthesis. Changes in the seeding efficiency of CFU-S after incubation was not studied and it can be suggested that this would increase survival due to the increase of seeding efficiency. However in this work the number of colonies derived from cells incubated with bone marrow conditioned media was not different significantly from the number of colonies derived

from cells incubated with medium. Therefore the decreasing number of colonies during suicide experiments is not due to the toxicity of CM or changes in seeding efficiency but due to the cells killed by cytosine arabinoside.

It is likely that these various factor(s) are parts of a complex network of cellular interactions which are providing an intergrated microenvironmental control of CFU-S behaviour the details of which are yet to be elucidated. Our studies suggest that certain populations of mononuclear phagocytes present in bone marrow from part of this regulatory complex.

SUMMARY AND CONCLUSIONS

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Following exposure to a dose of 0.5 Gy whole body X-irradiation the number of CFU-S per femur was not significantly changed. However the number of GM-CFC per femur was decreased to 14.8±1.1 to 29.8±1.5 percent of control levels between 30 minutes to 6 hours after exposure.

From the time course study of CFU-S entering into DNA synthesis following whole body X-irradiation, CFU-S were found to be triggered into DNA synthesis 30 minutes after exposure to 0.5 Gy dose. However at 6 hours after exposure the percentage was about control levels. At dose of 4.5 Gy the CFU-S were delayed in entering into DNA synthesis until 6 hours after irradiation. The presence of stimulator in bone marrow after irradiation was found to parallel the proliferative activity of CFU-S. When plugs of bone marrow were irradiated in-vitro at 0.5 Gy and 4.5 Gy doses the time of CFU-S entry into DNA synthesis was the same as following in-vivo irradiation.

From the dose response curve of CFU-S in DNA synthesis measured 2 hours after exposure, the percentage of CFU-S in DNA synthesis was increased when the dose increased and reached 30 to 50 percent at a dose of 0.5 Gy. Above this dose the CFU-S population was not stimulated at this time. However when the percentage of CFU-S in DNA synthesis was measured 6 hours after exposure the values were the same as control levels for doses less than 0.5 Gy and above this dose the values were 30 to 50 percent. Furthermore in this study, the CFU-S population obtained one hour after exposure to 1.5 Gy was shown not to respond as CFU-S from normal bone marrow.

The fast triggering of CFU-S into DNA synthesis at 0.5 Gy could be due to a cell surface membrane effect which resulted in changes in interaction between CFU-S and the environment. This might produce a signal which would stop the production of inhibitor and allow the production of stimulator. However this signal would be transient as there was no change in the CFU-S numbers. The delay in CFU-S entering into DNA synthesis at sub-lethal doses might be due to damage to the receptor for the stimulator molecules on

CFU-S which caused failure to respond to stimulator at early time after exposure.

The aplastic bone marrow where the haematopoietic cells in bone marrow were markedly depressed following 9.0 Gy whole body X-irradiation was shown to produce stimulator at day 1 to day 5 post irradiation. This suggests that stimulator producing cells were radioresistant and might be part of the haematopoietic environment. The depletion of $Thy1.2^+$ cells from regenerating bone marrow had no affect on stimulator production. However when Fc^+ and $Ia\text{-}2k^+$ cells were removed the production of stimulator was affected. Stimulator producing cells might be mononuclear phagocytes.

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